UNIVERSITÁ DEGLI STUDI DI NAPOLI

"FEDERICO II"

Dipartimento di Sanità Pubblica

Scuola di Dottorato in Morfologia Clinica e Patologica

XXVII Ciclo

Coordinatore: Prof.ssa Stefania Montagnani

Tesi di dottorato di ricerca

BIOMIMETIC SCAFFOLDS AND CARDIAC FIBROBLAST-DERIVED EXTRACELLLULAR MATRIX FOR STEM CELL-BASED MYOCARDIAL REGENERATION

RELATORE: **CANDIDATO:**

Prof.ssa Daria Anna Nurzynska **Dott.ssa Anna Maria Sacco**

ANNO ACCADEMICO 2013/2014

Credo di poter affermare che nella ricerca scientifica
né il grado di intelligenza né la capacità di eseguire
e portare a termine il compito intrapreso
siano fattori essenziali per la riuscita
e per la soddisfazione personale.
Nell'uno e nell'altro contano maggiormente
la totale dedizione e il chiudere gli occhi
davanti alle difficoltà: in tal modo possiamo affrontare
i problemi che altri,
più critici e più acuti, non affronterebbero.
Rita Levi-Montalcini

I believe I can state that in medical research
neither intelligence nor the capability
of accomplishing a task
is essential for personal satisfaction.
In both it is compulsory to ignore
inherent difficulties in order to tackle problems
that sharper and more critical people wouldn't face.
Rita Levi-Montalcini

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Abstract

Recent findings indicate that the number of cardiac primitive cells in the adult human heart increases significantly in pathological conditions. The fact remains, however, that those cells fail to accomplish cardiac tissue regeneration in chronic pathological conditions *in vivo*. Similarily, therapeutic stem cell delivery, either intravenous, intracoronary or intramyocardial, and activation, for instance by genetic modification or local growth factor injection, have yielded moderate and controversial results that make stem cell-based myocardial regeneration still merely an experimental approach to cardiac disease treatment. Adverse effects of underlying pathology, with cellular senescence and microenvironment modifications, might be responsible for such outcome.

The aims of the present research were to characterize cardiac primitive cells in the normal and pathological (ischemic cardiopathy) adult human heart, to produce and characterize cardiac fibroblast-derived extracellular matrix *in vitro*, and to characterize cardiac primitive cells cultured in the presence of this substrate in terms of their survival, proliferation, migration, and maturation. These findings should then lead to the identification of the biochemical signalling molecules, i.e. bioactive components of cardiac extracellular matrix, to be integrated in the scaffolding bioartificial materials and allow the evaluation of the bioactivity of scaffolds incorporating extracellular matrix proteins. Thus, the final aim of the study was to develop bioactive scaffolds populated with cardiac primitive cells for the regeneration of infarcted myocardial tissue, based on bioactive and tissue-specific

molecules, exerting the same biochemical signals of the natural extracellular matrix during heart development and regeneration.

Cardiac primitive cells and cardiac fibroblasts were isolated from samples of cardiac tissue derived from donor hearts and from hearts explanted due to ischemic cardiopathy in patients with end-stage heart failure. The population of cardiac primitive cells and the extracellular matrix deposited by cardiac fibroblasts *in vitro* were characterized by immunohistological methods, immunoblotting, immunoabsorbent enzymatic assay, or RT-PCR. Cardiac primitive cells from normal and pathological hearts were cultured in the presence of normal and pathological cardiac fibroblast-derived matrix, cardiac fibroblast-conditioned medium, or polyurethane scaffolds functionalized with gelatin or laminin-1 and their proliferation, apoptosis, migration and maturation were evaluated by BrdU incorporation assay, TdT assay, scratch wound assay and RT-PCR, respectively.

The results of the study highlighted the role of microenvironment in cardiac regeneration. Changes that take place in chronic pathological conditions should be taken into consideration when planning stem cell-based therapy. Cardiac fibroblast-derived matrix can be used for *in vitro* studies of the interactions between components of extracellular matrix and cardiac primitive cells responsible for cardiac self-renewal in normal and pathological conditions. The goal of regenerative medicine and tissue engineering to regenerate damaged myocardium can be achieved by further investigations into cell-matrix interactions at different stages of cardiac ischemic disease progression.

1. Introduction

1.1. Cardiac stem cells and cardiac primitive cells

Stem cells have been defined as a cell population capable of generating identical progeny through an unlimited number of cell divisions, while retaining the ability to respond to physiological demands by producing daughter cells committed to differentiation [1]. The presence of dividing stem cells has been observed in organs considered to be terminally differentiated, such as brain and heart [2]. Multipotent stem cells in adult tissues, including the human myocardium, have been identified by the expression of stem cell markers originally employed to identify cells of the hematopoietic lineage. Among these markers the main one is CD117, also known as c-kit in mice. It is a tyrosine kinase receptor found on stem cells, mast cells, germ cells, and melanocytes. The c-kit ligand, stem cell factor, is vital for normal hematopoiesis, melanogenesis, gametogenesis, and the growth and differentiation of mast cells. C-kit-expressing bone marrow-derived stem cells differentiate into blood and vascular endothelial cells and play a crucial role in the amplification and mobilization of progenitor cells [3]. Stem cells with the capacity to differentiate into three major cardiac cell types - cardiomyocytes, smooth muscle cells and endothelial cells have been described in both embryonic [4,5,6] and adult heart tissue [7]. These cells have been characterized by an array of membrane, cytoplasmic and nuclear antigens, yet the expression of different markers could be associated simply with the degree of stem cell differentiation. Despite different phenotype description in various studies, it is widely acknowledged that the cardiac hematopoietic lineage-negative (CD45-negative, CD34-

negative) putative stem cells express neither muscle nor endothelial cell markers at an undifferentiated stage [8], while their committed progenies express cell specific transcription factors and cytoplasmic proteins. In particular, progenitors and precursors were identified in the adult human heart by the expression of cardiac cell lineage specific markers. These included transcription factors such as myocyte enhancer factor 2C, (MEF-2C) and Nkx2.5 (in cardiomyocyte progenitors), GATA-6 (in smooth muscle cell progenitors) and external transcribed spacer (Ets-1, in endothelial cell progenitors), while the cytoplasmic proteins α-sarcomeric actin (α-SA) and ventricular α/β myosin heavy chain (MHC), smooth muscle actin (SMA), factor VIII (FVIII) and vascular endothelial growth factor receptor (VEGFR) were the markers of the precursors for cardiomyocytes, smooth muscle and endothelial cells, respectively [9].

Recent findings indicate that the number of CD117-positive cells in the adult human heart increases significantly in ischemic cardiomyopathy and pressure overload [10,11,12,13]. The fact remains, however, that those cells fail to accomplish cardiac tissue regeneration in chronic pathological conditions *in vivo*. Adverse effects of underlying pathology, with cellular senescence and microenvironment modifications, might be responsible for such outcome.

The above-mentioned evidences pointed to a shift in paradigm concerning the biology of the heart and put forward potential therapeutic strategies for the failing heart. Cardiac primitive cells seem to be a promising target in the therapy of acute and chronic heart disease, where cardiac regeneration may be accomplished by enhancing the normal

turnover of myocardial cells by stimulation of resident stem cells [14] or by transplantation of stem cells with cardiac differentiation potential derived from other sources [15]. Among mechanisms of the stimulation of cardiac regeneration the role of the injected cell - cytokine - resident cell axis has attracted attention of researchers and clinicians [16]. Several studies demonstrated that bone marrow-derived mesenchymal stem cells injected and incorporated into the myocardium released factors that acted in a paracrine manner to support local angiogenesis and activate tissue residing progenitor cells [17]. Interestingly, similar effects were exerted even by cell-free culture supernatant recovered from hypoxia-treated mesenchymal stem cell cultures [18]. Hence, determination of the growth factors and cytokines secreted by these cells and identification of their specific receptors on cardiac stem cells could provide more direct methods for boosting proliferation, survival and differentiation of cardiac progenitor cells. Since both the phenotype of CPCs and profile of bioactive cytokines in the local environment in which they accumulate differ between normal and pathological conditions, the characterization of cardiac tissue and cardiac stem cells in healthy heart and at different stages of heart failure should help in identifying the best approach for cardiac regeneration, possibly integrating cell transplantation with the stimulation of environmental factors that regulate biological properties of cardiac stem cells.

Stem cells are stored in niches formed by cellular and extracellular components that constitute the microenvironment in which stem cells are maintained in a quiescent state. After activation, stem cells replicate and migrate out of the niches to sites of cell replacement where they differentiate and acquire the adult phenotype. Niche homeostasis is

controlled by stem cell symmetric and asymmetric division, which preserves the proportion of multipotent stem and committed primitive cells within the parenchyma. In the niches, stem cells are connected to the supporting cells which anchor stem cells to the niche and modulate growth signals from the surrounding tissue [19]. Hence, the niche constitutes a dynamic entity in which the control of stem cell function depends on the complex interaction between intrinsic and extrinsic factors. According to one study [19], cardiac niches contained lineage negative cells nested together with cardiac progenitors and precursors. In close proximity to lineage negative cells, myocytes and fibroblasts were commonly visible. Within the niches, stem cells were connected to the supporting cells by gap and adherens junctions composed of connexins and cadherins, respectively. The recognition that junctional complexes are present between CSCs and myocytes or fibroblasts suggests that these cell types act as supporting cells within the cardiac niches. Conversely, endothelial cells and smooth muscle cells did not exert this role.

Niche and tissue microenvironment assure survival and control biological activity of stem cells and their progeny. Extracellular matrix, with growth factors stored within it, contributes to this microenvironment. Several cardiac pathologies, including myocardial ischemia, are associated with qualitative and quantitative alterations in matrix composition [20]. In the setting of increased wall stress, injury, or disease, the extracellular matrix can undergo a series of dynamic changes that at first lead to favorable chamber remodeling and functional adaptation, but over time can impair diastolic and systolic function due to excessive deposition of interstitial fibrous tissue. These pathological alterations in extracellular matrix structure/function are considered central to the evolution of adverse

cardiac remodeling and the development of heart failure. Therefore it seems reasonable that the role of extracellular matrix and, even more importantly, the effects of the modifications of its composition ongoing in pathological conditions should be studied and taken into consideration when planning the use of cardiac primitive cell-mediated tissue regeneration.

1.2. Cardiac extracellular matrix

Cardiac extracellular matrix is made of two basic structural organizations: the basal lamina, which surrounds individual myocytes and blood vessels, and the interstitial matrix which provides structural support for higher order cardiac myocyte organization as well as for larger blood vessels in the myocardium [21]. Exctracellular matrix, as a non-cellular component, is responsible for the physical maintenance of all cardiac cells, namely cardiomyocytes, fibroblasts, endothelial cells, and vascular smooth muscle cells. Extracellular matrix plays a role in numerous cellular processes and the concept that it has a passive role to play in cellular activity has been refuted. It provides not only essential physical scaffolding for the cellular constituents but also sends crucial biochemical and biomechanical cues that are required for tissue and cell morphogenesis, differentiation and homeostasis [22,23].

In heart tissue, extracellular matrix is essential for proper cardiac structural integrity and pump function, arranging a framework for myocytes, fibroblasts, and endothelial cells, and transmitting mechanical forces and signals to myocardial fibers [24]. Moreover, the extracellular matrix is an important mediator of growth-related factors and cytokines and

participates in modulating the cardiac phenotype during development and hypertrophy. Therefore, the disruption of extracellular matrix homeostasis is indeed a key factor for the progression of cardiac dysfunction [24].

Taking its structure into consideration, the extracellular matrix can be described as a network of multiple matrix proteins. These proteins provide the necessary support to cells and tissues forming the principal component of the extracellular matrix. Its constitutive proteins can be categorised as either structural or non-structural. Examples of structural proteins include collagens and elastin, while fibronectin, laminin and tenascin are instances of non-structural proteins, also known as glycoproteins. The fibrillar components of the extracellular matrix are organised into a recognisable three-dimensional structure. Various collagens form the framework of both the basement membrane and interstitial matrix, while the non-fibrillar proteins bind to this scaffold, communicating with surrounding cells via integrins [25]. Other important components of the extracellular matrix include growth factors and a family of matrix metalloproteinases (MMPs).

In short, the term cardiac extracellular matrix encompasses the following components:

- collagens (e.g., fibril-forming collagens and non-fibril forming collagens), the most abundant structural components of the extracellular matrix. These proteins are produced primarily by fibroblasts. Deposition of collagen indirectly modifies structural and functional properties of connective tissue within organs, which on one hand determines and guarantees the proper function of some tissues, e.g. cartilage and bone, but on the other

hand, impedes correct function of others, such as muscle or epithelium, due to interstitial fibrosis.

- glycoproteins (e.g., fibronectin, elastin, laminins, tenascins) and proteoglycans, essential in various extracellular matrix functions. Recently, proteins with a role that goes beyond the structural and mechanical support have been distinguished among other extracellular matrix components and termed matricellular proteins. This group encompasses proteins that modulate cell function by interacting directly with cells or by modulating the activity of soluble factors present in extracellular microenvironment, thus influencing cell migration, proliferation, and differentiation [26]. Moreover, these proteins present unique expression pattern, with high levels during organogenesis, virtual absence in normal adult tissue, and re-expression in response to injury and tissue regeneration. In the heart, osteopontin, osteonectin, thrombospondins, tenascin, and CCN family are the matricellular proteins identified so far [27]. Given these properties, extracellular matrix, and matricellular proteins in particular, can drive cardiac tissue regeneration, described in adult human heart in infarction or pressure overload [28].

- matrix metalloproteases (MMP-2 and MMP-9) that guarantee constant extracellular matrix remodelling. This constant brakedown process makes extracellular matrix a dynamic structure [25], capable of adaptation and response to factors and mechanisms activated in tissues in different physiological and pathological conditions.

- extracellular matrix receptors, mainly integrins. They conduce cell interaction with adhesive glycoproteins, including laminin and tenascin, and proteoglycans, allowing cell adhesion [25]. The interactions between extracellular matrix, cell membrane and

cytoskeleton through integrins might be particularly important during cardiac remodeling [29].

The structure, properties and functions of several extracellular matrix components with particular significance in cardiac tissue engineering is described in the following section.

1.2.1. Collagens

Collagen is the most abundant protein within the body and is found amassed in the extracellular matrix of connective tissues such as tendon and skin. It is the predominant form of structural protein found within the extracellular matrix, providing not only tensile strength but also playing a role in other cell processes such as adhesion and migration [30]. During development, collagen can be detected first (data from the chick embryo, [31]) in the incomplete basal lamina that is secreted beneath the epiblast, where it provides the substrate for the migration of cells during gastrulation. The organization of the cardiac collagen network in an organ is established shortly after birth and this extracellular matrix architecture persists in the normal adult heart. However, changes in the organization and composition of the extracellular matrix are a structural milestone in the development and progression of heart failure, irrespective of etiology.

Fibrillar collagen expression, synthesis and post-translational modification are fundamental roles of the fibroblast. To date, nearly 30 types of collagen have been distinguished [32]. Each protein contains three polypeptide (α) chains, displaying an extended polyproline-II conformation, a right-handed supercoil and a one-residue stagger between adjacent chains [33]. Each polypeptide chain has a repeating Gly-X-Y triplet in which glycyl residues

occupy third position and the X and Y positions are frequently occupied by proline and 4-hydroxyproline, respectively. These repeats allow the formation of a triple helix, which is the characteristic structural feature of the collagen superfamily. Each member of the collagen family contains at least one triple-helical domain (COL), which is located in the extracellular matrix, and most collagens are able to form supramolecular aggregates. Besides triple-helical domains, collagens contain non triple-helical (NC) domains, used as building blocks by other extracellular matrix proteins [34].

The classical fibril-forming collagens include collagen types I, II, III, V, and XI. These collagens are characterized by their ability to assemble into highly orientated supramolecular aggregates with a characteristic suprastructure [35]. In particular, collagen types I and III are predominant interstitial collagens in the heart and surround cardiac myocytes and the coronary microcirculation, providing structural integrity for the cardiomyocytes [29]. Type I collagen makes up approximately 85% and type III collagen 11% of total collagen in the heart [36]. The collagen type I triple helix is usually formed as a heterotrimer by two identical α1(I)-chains and one α2(I)-chain. The triple helix of type II collagen is composed of three a1(II)-chains forming a homotrimeric molecule similar in size and biomechanical properties to that of type I collagen.

Type III collagen is a homotrimer of three identical α1(III)-chains. It is an important component of reticular fibres in the interstitial tissue of the lungs, liver, dermis, spleen, and vessels. This molecule often contributes to mixed fibrils with type I collagen and is also abundant in elastic tissues [37]. Types V and XI collagens are formed as heterotrimers of three different α-chains (α1,α2,α3). It is remarkable that the α3-chain of type XI collagen is

encoded by the same gene as the α1-chain of type II collagen and only the extent of glycosylation and hydroxylation differs from α1(II). Although it is not finally sorted out, a combination of different types V and XI chains appears to exist in various tissues and they share similar biochemical properties and functions with other members of this family. Type V collagen typically forms heterofibrils with types I and III collagens and contributes to the organic bone matrix, corneal stroma and the interstitial matrix of muscles, liver, lungs, and placenta. It is also expressed in the heart and evidence supports its role in providing a core for fibrillar collagen assembly [38]. Type IV collagen is the most important structural component of basement membranes integrating laminins, nidogens and other components into the visible two-dimensional stable supramolecular aggregate. Other collagens (e.g. types VI, IX, XII and XIV) associate with fibril surfaces or (collagens types XII and XVII) are transmembranous proteins [25].

Recent studies of collagen receptors shed light on their expression and effects of their activation on cell biology. For a long time, integrins were considered to be the only class of cell surface receptors that could transmit signals into cells by binding extracellular matrix components. Recently, however, discoidin domain receptors (DDRs) with cytosolic tyrosine kinase activity have been identified, that, unlike other receptor tyrosine kinases which bind mostly soluble peptid-like growth factors, were activated by collagens [39]. Hence, cells contain at least two types of collagen receptors: integrins and DDRs. The collagen receptor integrin subfamily is composed of heterodimers $\alpha_1\beta_1$, $\alpha_2\beta_1$, $\alpha_{10}\beta_1$, and $\alpha_{11}\beta_1$. DDRs are the tyrosine kinase receptors of collagen expressed by epithelial (DDR1) and mesenchymal cells (DDR2). While integrin β_1 and DDR1 are both required for cell

adhesion on collagen, their roles in epithelial cell differentiation during development and disease progression seem to counteract each other, with integrin β_1 favoring transition to mesenchymal and DDR1 inducing differentiation towards epithelial phenotype [40]. Although not directly contrasting, but nevertheless still distinct effects, were observed upon activation of specific integrins in the presence of collagen [41]. The complexity of these interactions and their possible effects on biological properties of cells is further augmented by yet another variable: the expression of integrins and DDRs differs between physiological and pathological conditions. These changes can be considered a result of microenvironment modification but, in the light of the aforementioned functions of the collagen and collagen receptors, at the same time they themselves may determine, for instance, fibrosis or cancer progression. Interaction of fibrillar collagen with epithelial cells mediated by integrin beta1 can favour epithelial-mesenchymal transition [42]. In consequence, epithelial cells lose their polarity and adhesion, while acquiring migration and invasion capabilities. This process has been found to represent one of the mechanisms of tumour progression and interstitial fibrosis, but it also plays essential role in tissue and organ development [43]. Recently, epithelial-mesenchymal transition has been implicated in adult organ regeneration, as at least some populations of cardiac stem cells may derive from epicardial mesothelium [44].

Since collagen is the most abundant component of the extracellular matrix, a significant amount of effort has been spent on the use of collagen gels (most often from tropocollagen type I) for creating tissue constructs. When cells are added to collagen gels, the virtuous cycle starts, with the mechanical properties of construct being modified by biological

activity of cells being modified by mechanical properties of construct. Importantly, over the long term, original collagen is progressively degraded and additional components are secreted and incorporated into the matrix. All these processes are highly dependent on collagen concentration, medium composition, cell number, cell type and culture conditions (discussed in [45]). Collagen-based cellullarized scaffolds have been recently used in regenerative medicine, including skin replacement in full thickness burn wounds [46]. Other applications, such as bone substitutes or artificial blood vessels and valves, are being extensively investigated, in order to define the best combination of mechanical and biological properties of the constructs.

1.2.2. Fibronectin

Fibronectin is a dimeric glycoprotein (with the subunits that range in size from 230 kDa to 270 kDa) found in the extracellular matrix of most tissues, it is located within the basement membrane, that serves as a bridge between cells and the interstitial collagen meshwork and influences diverse processes including cell growth, adhesion, migration, and wound repair [23]. This major constituent of the extracellular matrix is deposited as an insoluble complex by polymerization of either the cellular dimer, synthesized and secreted locally, or the soluble in plasma circulating dimer synthesized in the liver. It is assembled into a fibrillar matrix in all tissues and throughout all stages of life [47].

Cell adhesion to fibronectin depends on the RGD (abbreviation for the tripeptide sequence of Arg-Gly-Asp) motif that binds to α5β1 integrin. Additionally, several other integrins were shown to bind to the RGD motif of fibronectin, including all members of the αV

subfamily, α8β1, α9β1 and the platelet-specific αIIbβ3 integrin. The integrin α5β1 is the principal receptor involved in the process of fibronectin matrix assembly which together with the RGD region of fibronectin promotes binding of cells to this protein [48]. The fibronectin matrix is associated with the actin cytoskeleton of cells through integrin activity. The functional properties of the fibronectin fibrillar matrix are diverse and represent a prime example of how extracellular matrix protein assembly functions. First, fibronectin fibrils possess binding sites for multiple matrix components, which are used to orchestrate the assembly of several other proteins, including collagen I and III, fibulin-1, fibrinogen, thrombospondin-1, latent Transforming Growth Factor-β (TGF-β) binding protein-1 (LTBP-1), decorin and biglycan. Second, fibronectin fibrils provide structural support for cell adhesion and similarly to the adhesion receptors, most notably integrins, transduce signals that promote cytoskeleton dynamics, cell migration, cell proliferation and apoptosis. Finally, fibronectin controls the availability of growth factors, for example by regulating their activation from latent complexes as shown for TGF-β [49].

Fibronectin expression is a critical factor in cardiac extracellular matrix, particularly in developing myocardium and in response to injury. Fibronectin levels have been found to be higher in neonatal hearts when compared to adult. In the cardiovascular system, fibronectin is synthesized by many cell types but not by the cardiomyocytes. Recently, expression of fibronectin was shown to influence cardiac progenitor cell response after myocardial infarction in adult mice [50].

1.2.3. Laminin

Laminin is one of the main components of the basement membrane. The molecule appears to weigh between 200 and 400 kDa and is composed of three disulphide linked chains that form characteristic cross shape. It is an heterotrimeric glycoprotein consisting in one α, one β, and one γ chain, in various combinations. Five α, three β, and three γ chains that represent distinct gene products have been identified in vertebrates. Twelve distinct laminin isoforms have been isolated and the trimers are named according to their trimeric composition of α, β, and γ chains [51].

The largest chain is the α-chain, which contains the long arm on the C-terminal end and a short arm on the N-terminal end. The short arm of α chains displays great diversity in length. The C-terminal end of the long arm consists of the LG 1-5 domains, which are involved in interactions with cellular receptors such as integrins and dystroglycans, with proteins anchored in the plasma membranes of cells or microorganisms, thereby relaying biochemical and mechanical signals between intracellular and extracellular molecular networks. The N-terminal end of the short arm is also capable of binding to integrin receptors, although it is more associated with polymerization of the molecule [52]. The β and γ chains are involved in interactions with molecules in the extracellular matrix. Notably, the C-terminal region of laminin β chains may modulate the integrin binding affinities of laminins and integrin-mediated interactions of laminins are important for several cellular activities inasmuch as they activate specific signaling networks governing adhesion, migration, differentiation [53]. Some not yet well-defined parts of laminins are thought to interact with small molecules such as growth factors and cytokines. This

function is thought to be important for sequestration and storage of these small molecules and for regulating their distribution, activation, and presentation to cells.

Laminin isoforms are synthesized by a wide variety of cells, such as epithelial cells, smooth, skeletal, and cardiac muscle cells, endothelial cells, in a tissue-specific manner. Laminins have astonishing variety of effects on adjacent cells, including cell adhesion, cell migration, and cell differentiation. They exert their effects mostly through integrins, many of which recognize laminins, via the integrin binding domain residing predominantly in the α chain [54]. At least eight different integrins (α1β1, α2β2, α3β1, α6β1, α6β4, α7β1, α9β1, αvβ3) can bind to laminin [51]. The biological effects of the laminins are to a large extent mediated by surface receptors that bind laminin matrices to intracellular signaling pathways. Laminin-1 (α1β1γ1) is the first extracellular matrix protein to be expressed during embryonic development, and it has been observed that heart organogenesis does not proceed in the absence of this protein [55]. The absence of laminin-2 (α2β1γ1), an isoform typical of muscle tissue, causes congenital muscular dystrophy with cardiac involvement [56]. In the adult heart, laminin-1 and laminin-2 were found to protect from apoptosis and stimulate proliferation of CPCs *in vitro* [10].

1.2.4. Tenascin

Tenascins are a family of multimeric glycoproteins characterized by an N-terminal globular domain and heptad repeats, which facilitate multimerization; one or more tenascin-type epidermal growth factor (EGF)-like repeats; a series of fibronectin type III domains, and a C-terminal fibrinogen-related domain [57]. There are six isoforms of the tenascin gene

products: tenascin-C, X, R, Y, W, and N. Two members of the tenascin family, tenascin-C and -X, modulate cell migration, adhesion and growth [58]. These two isoforms are expressed in connective tissues, tendons, dermis, heart, kidney, vascular smooth muscle and by astrocytes and Schwann cells during neural development.

The complex domain structure of the tenascins predicts that the molecule is capable of interacting with a variety of extracellular matrix proteins. This indeed appears to be the case. Cell surface receptors for tenascins include integrins, cell adhesion molecules of the Ig superfamily, a transmembrane chondroitin sulphate proteoglycan (phosphacan) and annexin II. Tenascin-C also interacts with extracellular proteins such as fibronectin and the lecticans, a class of extracellular chondroitin sulphate proteoglycans including aggrecan, versican, and brevican. Furthermore, tenascins are recognized and cleaved by extracellular matrix proteases including serine proteases and matrix metalloproteases [25].

The patterns of expression of tenascins are rather complex. In particular, during embryogenesis the rate of tenascin synthesis changes significantly. In combination with the effects of tenascin on cell behaviour, this indicates that tenascin in the extracellular matrix might have an important function during morphogenesis in determining whether or not specific cells will adhere to the matrix, and in doing so receive cues which modulate cell differentiation, migration, proliferation, or apoptosis [25]. Tenascins are not expressed in normal adult tissues, however, as typical of matricellular proteins, they re-appear in a variety of pathological situations in response to some growth factors or mechanical stress

applied to cells *in vitro* or tissues *in vivo*. Tenascin-C expression is found in wound healing and in neovascularization accompanying, for example, tumour growth.

Specific roles of Tenascin-C in heart morphogenesis have long been anticipated based on its strictly regulated temporal expression at specific sites closely associated with cell migration, especially fibroblast migration and epithelial-mesenchymal/mesenchymal-epithelial transition. Tenascin-C is implicated in recruitment of cardiac myofibroblasts [59], differentiation of cardiomyocyte precursors, valve and coronary vessel formation. Following myocardial infarction, it can promote myocardial repair and prevent ventricular dilation by recruiting myofibroblasts to the site of injury and enhancing collagen fiber contraction. On the other hand, tenascin-C may also promote adverse myocardial remodeling. This protein has been shown to up-regulate MMP-2 and MMP-9 expression in a number of cell types, enhance inflammatory responses through activation of NFκB and cytokine upregulation, and inhibit the strong linkages that occur between cardiomyocytes and connective tissues [60]. Although these functions serve to clear damaged tissue and release residual cardiomyocytes from connective tissue for subsequent cell rearrangement, they may also contribute to progressive degradation of extracellular matrix and slippage of myocytes within the myocardial wall, ultimately resulting in left ventricle wall thinning and dilation. Accordingly, high serum levels of tenascin-C in patients following myocardial infarction have been associated with a greater incidence of adverse cardiac remodeling and poor prognosis, supporting the view that excessive and sustained increments of tenascin-C could lead to detrimental myocardial remodeling [61].

Another and the largest member of the same family is tenascin-X. It is expressed much more widely than other tenascin members, predominantly in heart, skeletal muscle and gut. It has been shown that tenascin-X in combination with vascular endothelial growth factor-B enhances endothelial cell proliferation [62]. It plays a central role in organizing and maintaining the structure of tissue supporting muscles, joints, organs, and skin. Tenascin-X causes also the formation of collagen fibrils and regulates the structure and the stability of elastic fibers within the extracellular matrix [63].

1.2.5. Matrix Metalloproteinases (MMPs)

MMPs are zinc-dependent endopeptidases that normally contribute to tissue remodeling in several physiological processes including development, hormone-dependent tissue remodelling, and tissue repair [64]. MMPs are the key group of protease enzymes involved in disintegration of the extracellular matrix and are effective in their capacity to break down the matrix, as part of the on-going remodelling of the extracellular matrix. According to the extracellular protein substrate, myocardial MMPs are classified into 4 groups: collagenases (MMP-1 or interstitial collagenase, MMP-8 or neutrophil collagenase, and MMP-13 or collagenase 3), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10 and MMP-11) and membrane-type MMPs (MT-MMPs). Gelatinases, MMP-2 and MMP-9 are the most abundant MMPs in the heart, as they are found in cardiac myocytes, fibroblasts and endocardial and subendocardial layers. Gelatinases degrade fibrillar collagen (types I, II and III) as well as collagen type IV, fibronectin and laminin. MMP-1, MMP-8 and MMP-13, as indicated by their name (collagenases), have a high affinity for fibrillar collagen, as well as other extracellular matrix proteins, such as proteoglycans, aggrecan,

versican and perlican. Stromelysin substrates include membrane proteins, elastin and proteoglycans. Finally, MT-MMPs degrade all membrane proteins as well as other extracellular matrix components [64].

Matrix metalloproteinases are synthesized as inactive zymogens and secreted into the extracellular space as pro-enzymes, by both cardiac myocytes and fibroblasts. This proenzyme form needs to be activated by proteolytic removal of its pro-domain in order to induce proteolysis of other proteins. MMP expression is rapidly induced by a variety of cytokines and growth factors that participate in events associated with tissue remodelling; however, MMP activity is controlled at several levels, including transcription, proteolytic activation, localization and interaction with inhibitors. Recent evidence suggests that secreted MMPs localize at least transiently to the cell surface by interacting with cell surface adhesion receptors and proteoglycans. Importantly, MMP proteolytic activity can activate latent secreted growth factors, e.g. MMP-2 and -9 can activate TGF-β, while several MMPs can release IGF from IGFBPs, and cell surface growth factor precursors, including TGF-α and EGF [65]. Thus, from being thought to primarily provide a mechanism to degrade extracellular matrix proteins and regulate mechanical resistance to cell migration, MMPs are now being recognized as major regulators of normal cell physiology and cell-matrix interactions.

Many MMPs participate in the physiological remodeling after a tissue injury, however overexpression of these enzymes and an excess extracellular matrix turnover can also have unfavorable effects. It can be illustrated by the events taking place after acute coronary

artery occlusion leading to cardiomyocyte death. An inflammatory cascade is triggered with activation of several pro-inflammatory cytokines, including IL-1 and TNF-a that induce migration and proliferation of leukocytes in the infarct region. In this inflammatory stage, proinflammatory cytokines promote activation of MMPs and extracellular matrix degradation. MMPs do not only degrade extracellular matrix but also regulate release and activation of several cytokines and growth factors, including TGF and VEGF thus, participating in the regulation of neovascularization. Although the presence of active MMPs is essential for the healing process after acute myocardial infarction, excessive degradation of extracellular matrix by MMPs resulted in an adverse remodeling and a marked LV dysfunction, thereby increasing the risk of complications such as congestive heart failure, aneurysm formation and infarct rupture. Indeed, the MMP-8 and MMP-9 levels were significantly higher in human hearts with ruptured infarcts than in control myocardial infarct tissue [66].

1.2.6. Integrins

The integrin family consists of a large multi-adhesive extracellular matrix molecules that bind different proteins, including extracellular matrix molecules, growth factors, cytokines, and MMPs. They are a major class of receptors involved in cell adhesion that control development. Beside mediating cell adhesion, integrins transmit signals across the plasma membrane that regulate cell migration, cell survival and growth and are implicated in pathogenesis of several pathologies, such as cancer and thrombosis [67].

Integrins, heterodimeric type I transmembrane proteins, consist of α and β subunits. The family is composed of 18 α subunits and 8 β subunits that can assemble into 24 different heterodimers. The integrin subunits range in size from 80 to 180 kDa. Each subunit contains a single transmembrane domain (TMD) and a short cytoplasmic tail. The integrins can be grouped based on ligand-binding properties or their subunit composition. In the cardiac myocytes, the integrin heterodimers most highly expressed are α1β1, α5β1 and α7β1, which are predominantly collagen, fibronectin and laminin binding receptors, respectively [68]. A key facet of outside-in signaling, relevant to the extracellular matrix, is the ability of integrins to transduce mechanical information into biochemical signals. Indirect activation by specific integrin expression and clustering can transmit extracellular biochemical inputs along the intracellular signalling pathways that regulate cell proliferation, survival, and migration. The expression profile of integrin subunits in the same cell population can change in qualitative and quantitative manner depending on microenvironmental conditions. As a result, extracellular matrix can influence cell and tissue development and overall cellular function.

1.3. Tissue engineering

Tissue engineering is a concept that first emerged in the early 1990s to provide solutions to severe injured tissues or organs. Robert Langer and Joseph P. Vacanti defined tissue engineering as "an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ" [69]. Since then, two main strategies have been adopted:

cell-based, where cells are manipulated to create their own environment before transplanted to the host, and scaffold-based, where an extracellular construct is created to mimic *in vivo* structures.

Basic component of an engineered tissue is a scaffold that acts as a temporary template, guiding cell organization, growth and differentiation and providing structural stability and a 3D environment where cells can produce new biological tissue [70]. Cells are cultured *in vitro* under precisely controlled culture conditions on a porous three-dimensional material that acts as scaffold for cell growth and proliferation. Once being implanted in the human body, the scaffold is eventually bioresorbed and the space occupied by it is replaced by new tissue produced by cells [71]. The officially accepted definition considers a scaffold as a "support, delivery vehicle or matrix for facilitating the migration, binding or transport of cells or bioactive molecules used to replace, repair or regenerate tissues" [72]. While the extracellular matrices are the native scaffolds of the human body, tissue engineering scaffolds are intended to provide the temporary structural support and biochemical or biomechanical signals needed to transform unassociated cells into human organs.

Scaffolds may be created from various types of materials, including natural and synthetic polymers and inorganic substances. These materials should be biocompatible and cell "friendly" (i.e. they must not elicit harmful response from the biological environment and should enhance cell adhesion and survival both *in vivo* and *in vitro*), have mechanical properties consistent with those of the tissue it replaces, degrade at a rate matching that of new tissue formation, be bioresorbable, have proper surface properties to promote cell

adhesion, migration, proliferation and differentiation in order to obtain an entirely colonized 3D cell construct as well as to promote extracellular matrix formation; last but not least scaffolds should have the optimum architectural properties in terms of pore size and permeability in order to allow efficient delivery of nutrients and removal of metabolites [73]. In order to meet these guidelines in scaffold configuration, many classes of biomaterials have been pursued [74]: metals (Ti and its alloys, stainless steels, Au, Ag, Co-Cr alloys are used for joint replacements, bone plates and screws, dental implants, surgical instruments), ceramics (aluminium oxides, calcium phosphates, hydroxyapatite, bioglasses are used for dental implants, femoral heads, coating of orthopaedic devices), natural polymers derived typically from the extracellular matrix (proteins such as collagen, gelatin, fibrin, starch; polysaccharides, such as hyaluronic acid, alginate, chitin; and bacterial polyesters, such as polyhydroxybutyrate, polyhydroxyvalerate are widely used to produce scaffolds either as pure materials or in combination with synthetic polymers or inorganic substances), synthetic polymers (e.g. polyammides, silicon rubber, polyesters, polyurethanes, polytetrafluoroethylene, polymethylmetharylate, PGA, polylactic acid, and poly-E-caprolactone have been extensively used in tissue engineering applications and for sutures, blood vessels, catheters, devices for drug delivery, contact lenses). In particular, polyurethanes are a large group of polymers widely used in the biomedical and industrial field. Biocompatible polyurethane (PU) was synthesized from degradable and biocompatible building blocks: PCL diol, BDI and L-lysine ethyl-ester-dihydrochloride chain extender. This formulation was previously demonstrated to produce biomaterials as optimal substrates for myoblast adhesion, spreading and proliferation [75]. Finally, hybrid scaffolds are the combination of synthetic and biologic materials to form a scaffold that

uses the advantages of both. These scaffolds can be made in a variety of ways, such as coating biologic material onto a synthetic one, electrospinning a biologic and synthetic material together, or using biologic microspheres with a synthetic patch [76].

Once a scaffold has been formed, several strategies can be applied to immobilize short peptide sequences on material surfaces, including covalent tethering of the functional groups, plasma treatment, functionalizing the substrate with amine or thiol groups to enhance peptide tethering, and photopolymerizing polymers with the peptide sequences [77]. Indeed, nowadays the main objective of tissue engineering is to make the scaffold as much biomimetic as possible, not only in terms of three-dimensional structural features and mechanical properties, but also from a chemical point of view. The dynamic composition of extracellular matrix acts as a reservoir for soluble signaling molecules and mediates signals that control migration, proliferation, and differentiation of cells during growth, development, wound repair, and tissue regeneration. These microenvironmental signals are generated from growth factors, cell- extracellular matrix, and cell-cell interactions, as well as from physical, chemical and mechanical stimuli. Consequently, approaches to tissue engineering focus on the need to provide cell populations with signals to promote cell proliferation and differentiation [78]. For this reason, the extracellular matrix is inevitably taken as a benchmark. Since extracellular matrix protein fibers (with diameter in the range of 50–500 nm) are 1-2 orders of magnitude smaller than the cells, they are able to contact directly with cells in three dimensional orientations which may be a crucial factor in the selection for scaffold coating. The incorporation of those proteins is considered a valid approach to confer bioactivity to the scaffold and it is commonly achieved through either

bulk or surface functionalization. In the bulk functionalization, biomolecules are incorporated within the polymer matrix and they are released in the surrounding environment either by diffusion or via scaffold degradation [79]. The surface of scaffolds can be covered with peptides or proteins by plasma-mediated grafting [80].

Biological scaffolds composed of extracellular matrix or covered with a single protein have been used for the repair of a variety of tissues including skin, articular cartilage, tendons, bone, bladder, esophagus, trachea and myocardium, often leading to tissue-specific constructive remodeling with minimal or no scar tissue formation. In particular, myocardial tissue engineering has been proposed as an option to replace the scarred non-contractile fibrous tissue formed post-infarction. Such approach offers the possibility to combine cells and extracellular matrix and thereby provides a mechanical support to the diseased myocardium, the proper environment for transplanted cells and increased delivery efficiency [81]. The basic myocardial tissue engineering paradigm is to seed cells capable of forming cardiomyocytes onto a biocompatible material *in vitro*, followed by implantation of the construct on or in the infarcted region of the failing heart. The grafted tissue will in turn direct new tissue formation as the cells integrate with the native tissue while the scaffold degrades over time [79]. To reach this goal, the incorporation of extracellular matrix-derived peptides and proteins into biomaterials has been proposed to mimic biochemical signals [82]. The use of whole proteins, such as fibronectin and laminin, which are cell adhesion proteins of the extracellular matrix, is limited by their low stability and availability, and their high cost [83]. Alternatively to whole heavy proteins,

synthetic fragments of fibronectin, vitronectin, and stromal derived factor-1 were employed as potential adhesive sequences for cardiomyocytes.

Recently, new polyurethanes have been synthesized, incorporating extracellular matrix peptides and bioactive molecules (growth factors). Aminoacid sequences have been introduced in polyurethane chains to control the enzymatic degradation process of the resulting scaffolds [84]. The key advantage of this polymer is its elastic behaviour, in that it is able to withstand strong deformation forces and to return to its original size upon removal of stress. Hence, the use of elastomeric polyurethane circumvents the problems related to material stiffness and myocardium contractility. In a recent work, polyurethane scaffolds for myocardial tissue engineering were obtained using melt-extrusion technique. Bi-layered scaffolds 0°/90° lay-down pattern were fabricated with highly reproducible quality of the computer-designed architecture, demonstrating polyurethane suitability for melt-processing. Human CPCs were found to adhere on the scaffolds showing a spread geometry and retaining their viability and proliferation potential [84]. Optimization of scaffold geometry and surface chemical composition is in progress to obtain scaffolds with the best properties for myocardial tissue engineering.

2. Scope of the study

Recent discovery of tissue-resident adult stem/progenitor cells has led to an explosion of interest in the development of novel stem cell-based therapies for improving the regenerative capacity of endogenous immature cells or transplanted cells for the repair of tissues damaged by disease. The fact remains, however, that those cells fail to accomplish cardiac tissue regeneration in chronic pathological conditions *in vivo*. Similarily, therapeutic stem cell delivery, either intravenous, intracoronary or intramyocardial, and activation, for instance by genetic modification or local growth factor injection, have yielded moderate and controversial results that make stem cell-based myocardial regeneration still merely an experimental approach to cardiac disease treatment. Adverse effects of underlying pathology, with cellular senescence and microenvironment modifications, might be responsible for such outcome.

The adult heart is mainly composed of terminally differentiated cells, but it is not a terminally differentiated organ since it harbors stem cells supporting its regeneration. Cardiac stem cells exist *in vivo* in a specialized microenvironment which assures their survival and regulates their proliferation and differentiation. The supporting cells, extracellular matrix with growth factors stored within it, and physical characteristics of the surrounding structures, all contribute to cardiac niche. The concept of stem cell niche sees the microenvironment as the major determinant of stem cell maintenance and explains the dependence of stem cells upon their microenvironment. In the niche, stem cells interact

with various extracellular matrix components. With the myocardial tissue engineering in mind, the extracellular matrix would be, by definition, an ideal bioactive scaffold construct.

In the last decade, several tissue engineering approaches have been proposed for the regeneration of infarcted myocardial tissue, with the development of cardiac patches being one of them. Cardiac patches have been designed to provide initial mechanical support to the damaged tissue, thus reducing remodelling, to support the attachment, proliferation, migration and differentiation of both endogenous and exogenous cells and to gradually degrade over time as the cells form their own extracellular support structures. The choice of the scaffold biomaterial plays a key role in tissue engineering strategies. Particularly, the scaffold should exhibit a biomimetic behaviour with respect to the tissue to be regenerated, in order to direct the organization, growth and differentiation of cells.

New findings in stem cell biology have suggested that stem cells are a potential source of heart muscle cells and can be used to rebuild or replace damaged heart tissue. The most common approach in regenerative medicine is to grow cells *in vitro* on scaffolds to get the required three-dimensional tissues. Given these premises, the aim of the present research was to characterize cardiac primitive cells in the normal and pathological heart, to produce and characterize cardiac fibroblast-derived extracellular matrix in vitro, and to characterize cardiac primitive cells cultured in the presence of this substrate in terms of their survival, proliferation, migration, and maturation. These findings should then lead to the identification of the biochemical signalling molecules, i.e. bioactive components of cardiac extracellular matrix, to be integrated in the scaffolding bioartificial materials and allow the

evaluation of the bioactivity of such scaffolds incorporating extracellular matrix proteins. Thus, the final aim of the study is to develop bioactive scaffolds, populated with cardiac primitive cells, for the regeneration of infarcted myocardial tissue, based on bioactive and tissue-specific molecules, exerting the same biochemical signals of the natural extracellular matrix during heart development and regeneration.

3. Materials and methods

3.1. Materials

Cardiac tissue samples were obtained from normal and pathologic human hearts. Samples of atrial appendages from normal hearts (n=24, mean age 50.4±4.1 years, 14 males, 10 females) were collected from the donor heart waste fragments, i.e. tissue trimmed off from the heart while adjusting atrium size and form at the time of organ transplantation. Pathological samples were taken from the corresponding region of explanted hearts of patients with end-stage heart failure due to ischemic cardiomyopathy undergoing heart transplantation (n=22, mean age 55.8±3.1 years, 16 males, 6 females, mean ejection fraction 25±1%). Specimens were collected without patient identifiers in accordance with the protocols approved by Monaldi Hospital.

3.2. Isolation of fibroblasts and cardiac primitive cells

Cardiac tissue samples were dissected, minced, and enzymatically disaggregated by incubation in 0.25% trypsin and 0.1% (w/v) collagenase II (Sigma-Aldrich, St. Louis, MO, USA) for 30 minutes at 37^{o}C. The digestion was stopped by adding a double volume of Hank's balanced salt soltution (HBSS) supplemented with 10% fetal bovine serum (FBS). This preparation was further disaggregated by pipetting and tissue debris and cardiomyocytes were removed by sequential centrifugation at 100g for 2 minutes, passage through 20μm sieve, and centrifugation at 400g for 5 minutes. Cell population was seeded

on culture dishes in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (DMEM/F-12, Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), basic fibroblast growth factor (Peprotech, Rocky Hill, NJ, USA), glutathione (Sigma-Aldrich), penicillin and streptomycin (Life Technologies, Paisley, UK). Cell population was cultured for time interval ranging from 1 to 2 week. Once the adherent cells were more than 75% confluent, they were detached with 0.25% trypsin-EDTA (Sigma-Aldrich) and cell suspension was used for isolation of fibroblasts and cardiac primitive cells by immunomagnetic cell sorting (Miltenyi Biotec Bergisch Gladbach, Germany). In particular, fibroblasts were purified by positive selection with anti-fibroblast MicroBeads and passage through MS columns placed in magnetic field, followed by incubation of the collected negative fraction with anti-human-CD117 MicroBeads and positive selection of CD117-positive cardiac primitive cells.

3.3. Extracellular matrix deposition and denudation *in vitro*

Cardiac fibroblasts were seeded (15×10^3cells/cm^2) on gelatin-coated culture plates in DMEM supplemented with 10% FBS and cultured in confluent state for up to 21 days, allowing for extracellular matrix deposition, according to the modified and optimized protocol used for the culture of cardiac primitive cells. Successively, fibroblasts were removed by 5-minute incubation with a solution of 0,5M NaCl and 10mM Tris-base, followed by short incubation with 1% SDS. The denudation process was observed at an inverted phase contrast microscope (Olympus Italia, Segrate, Italy). When cells were no

longer discernible, the solution was diluted and removed, followed by gentle washing of culture plates with PBS.

3.4. Culture of CD117-positive cardiac primitive cells

CD117-positive cardiac primitive cells, isolated and purified as described above (section 3.2), were plated at a density of $4x10^3$ cells/cm^2 in DMEM/F-12 (Sigma-Aldrich) supplemented with 10 % FBS (Sigma-Aldrich), basic fibroblast growth factor (Peprotech), glutathione (Sigma-Aldrich), penicillin and streptomycin (Life Technologies). CD117-positive cells at first or second passage were used for characterization and subsequent experiments.

3.5. Characterization of cardiac extracellular matrix and cardiac primitive cells

3.5.1. Immunofluorescent staining

Fibroblast-derived cardiac matrix obtained *in vitro* was fixed in 4% paraformaldehyde for 20 minutes at room temperature. After blocking with 10% donkey serum, plates were incubated with primary antibody against fibronectin (rabbit polyclonal anti-human, Sigma-Aldrich), collagen IV (mouse monoclonal anti-human, Sigma-Aldrich), tenascin-C (rabbit polyclonal anti-human, Santa Cruz Biotechnology, Dallas, TX, USA), or laminin (mouse monoclonal anti-human, Sigma-Aldrich) and specific secondary antibodies conjugated with fluorescein or rhodamine (Jackson ImmunoResearch Europe, Newmarket, UK); F-actin was stained with rhodamine phalloidin (Sigma-Aldrich).

Cardiac primitive cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature and incubated with 10% donkey serum. Cells were incubated with primary anti-human antibodies against Ki67 (monoclonal mouse, Novocastra Laboratories Ltd., Newcastle, UK), Notch (monoclonal mouse, Abcam, Cambridge, UK), α-sarcomeric actin, von Willebrand factor (vWF), smooth muscle actin, (all mouse monoclonal, Sigma-Aldrich), Nkx 2.5 (rabbit polyclonal, Chemicon), Ets-1 (rabbit polyclonal, Santa Cruz Biotechnology), or GATA-6 (goat polyclonal, Santa Cruz Biotechnology), followed by secondary antibodies conjugated with fluorescein or rhodamine (Jackson ImmunoResearch Europe). Nuclei were counterstained with DAPI (Merck Millipore).

Stained area of culture dish was mounted in Vectashield (Vector Labs). Microscopic analysis was performed with a Leica DMLB microscope equipped with epifluorescence EL6000 system (Leica Microsystems). Pictures were taken with digital camera connected to the microscope (Leica DFC345FX) and then merged with the software Leica Application Suite.

3.5.2. Electrophoresis and immunoblotting

Protein extracts were prepared from cardiac matrix (Cm) secreted by fibroblasts derived from normal (CF-N) and pathological (CF-P) hearts. Cardiac matrices were harvested by scraping in PBS and then incubated on ice in a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 250 mM NaCl, 0.1% Triton X-100 supplemented with proteases inhibitors (1 mM DTT, 2 mM PMSF, 2 µg/ml aprotinin and 10 µg/ml leupeptin). Lysates were centrifuged and protein concentration in the supernatants was determined using

Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA). Albumin from bovine serum (BSA, Sigma-Aldrich) was used to construct the standard curve. Lysates containing 30μg of proteins were size-fractionated by electrophoresis on 8% SDS-polyacrylamide gel and transferred onto a polyvinylidene difluoride (PVDF) membrane (Merck Millipore). Molecular weight markers were loaded onto each gel as a weight indicator. The membranes were blocked and then incubated with one of the following antibodies: tenascin-X, lamininα1, lamininα2, fibronectin, and collagen I, or α-actinin (for loading control), followed by horseradish peroxidase-labelled secondary IgG (all from Santa Cruz Biotechnology).

In the same manner, protein extracts were prepared from CD117-positive cells isolated from normal and pathological hearts. Lysates containing 30μg of proteins were size fractionated by electrophoresis on 8% SDS-polyacrylamide gel and transferred onto a nitrocellulose membrane. Lysates of the whole cardiac tissue and molecular weight markers were loaded onto each gel as a positive control and weight indicator, respectively. The membranes were blocked and then incubated with one of the following antibodies: myosin light chain 1, myosin light chain 2, factor VIII, smooth muscle actin, or α-actinin (for loading control), followed by horseradish peroxidase-labeled secondary IgG (all from Santa Cruz Biotechnology).

Antibody binding was visualized by chemiluminescence (Amersham Biosciences, Piscataway, NJ, USA) and autoradiography (Eastman Kodak Company, Rochester, NY,

USA). The intensity of individual bands was determined using ImageJ software (http://rsb.info.nih.gov/ij/).

3.5.3. RNA isolation and PCR-Array

Total RNA was isolated from cardiac CD117-positive cells derived from normal hearts using standard RNA extraction protocols (NucleoSpin RNA II, Macherey-Nagel, Duren, Germany). The pooled RNA from this reference was used for competitive hybridization against the RNA from samples of heart of patients with end-stage ischemic cardiomyopathy obtained in the same manner. All RNA samples were quality checked and were considered suitable for gene expression profiling experiments. The process of gene expression analysis using PIQOR^{TM} Stem Cell Microarrays was performed by Miltenyi Biotec. To exclude labeling bias, the researchers remained unaware as to the protocol of the experiment and the real significance of the samples received for analysis. Sample labelling was performed using Cy3 (green) for normal and Cy5 (red) for pathological heart samples. Following the overnight hybridization to PIQOR^{TM} Stem Cell Microarray Human Antisense in the a-Hyb^{TM} Hybridization Station, fluorescence signals were detected using the laser scanner ScanArray^{TM} Lite (PerkinElmer, Waltham, MA, USA). Data were analyzed by Gene Functional Classification Tool available from Database for Annotation, Visualization, and Integrated Discovery [85].

The same samples of RNA were also analyzed with Human TGFb/BMP Signaling Pathway RT^2 Profiler^{TM} PCR-Array (SA Biosciences, Frederick, MD, USA), according to the manufacturer's protocol. For the cDNA synthesis, 1μg of total RNA was used with RT^2

First Strand Kit. Next, the cDNA was mixed with RT^2 SYBR Green Mastermix and real time RT-PCR was performed. The relative expression of the products was determined from raw data using ΔΔCt method.

3.5.4. RT-PCR and Real Time RT-PCR

Total RNA was extracted from normal and/or pathological cardiac primitive cells plated on control plates, cardiac fibroblast-derived matrix-covered plates and polyurethane scaffolds using the RNeasy RNA Isolation kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. The isolated RNA was dissolved in RNase-free water, and the final concentration of RNA was determined in a Qubit fluorometer (Invitrogen). A total amount of 100ng RNA from cells cultured on scaffolds and 1 μg of RNA from cells plated on culture dishes was retrotranscribed into cDNA with QuantiTect Reverse Trascription Kit (QIAGEN). Total RNA was quantified with a spectrophotometer and the quality was evaluated by gel electrophoresis with ethidium bromide staining. All primers (reported below) were designed with Primer3 software (http://frodo.wi.mit.edu).

Gene	Forward Primer	Reverse Primer	Amplicon Length (nt)
GAPDH	5'-CACCATCTTCCAGGAGCGAG-3'	5'-TCACGCCACAGTTTCCCGGA-3'	372
NKX2.5	5'-CCTCAACAGCTCCCTGAC-3'	5'-CTCATTGCACGCTGCATA-3'	162
α-SA	5'-TCGGGACCTCACTGACTA-3'	5'- GGGCTGGAAGAGTGTCTC-3'	282
VEGFR2	5'-GATGTGGTTCTGAGTCCGTCT-3'	5'- CATGGCTCTGCTTCTCCTTTG-3'	560
FVIII	5'-CAGCCTCTACATCTCTCAGTT-3'	5'-ATGCGAAGAGTGCTGCGAATG-3'	210
GATA6	5'- GCCCCTCATCAAGCCGCAGAA-3'	5'- TCTCCCGCACCAGTCATCACC-3'	378

RT-PCR was carried out using 5Prime HotMasterMix (5Prime, Hamburg, Germany) and RNA expression was analyzed by comparing the control transcript (GAPDH) and the transcript of interest when their amplification was in the exponential phase. The PCR products were size-fractionated by electrophoresis in 2% agarose gel. The DNA bands were visualized and photographed under UV light exposure with FireReader XS D-55 imaging system equipped with 1D software (UVItec Limited, Cambridge, UK). The intensity of individual bands was determined using ImageJ software (http://rsb.info.nih.gov).

Real Time RT-PCR was performed using RealMasterMix SYBR ROX (5Prime) according to manufacturer's protocol. DNA amplification was carried out using Mastercycler ep realplex^{4S} (Eppendorf), the thermal cycling conditions included an initial denaturation for 2 min at 95 °C and 40 cycles consisting of a denaturation step at 95 °C for 15 sec, an annealing step at 58 °C for 15 sec and an extension step for 30 sec at 68 °C. The detection was performed by measuring the binding of the fluorescence dye SYBR Green I to double-stranded DNA. All samples were tested in triplicate with the housekeeping genes (GAPDH and RPL13A) to correct for variations in RNA quality and quantity. Melt curve analyses were conducted to assess uniformity of product formation, primer dimers formation, and amplification of non-specific products. Linearity and efficiency of PCR amplification were assessed using standard curves generated by increasing amounts of cDNA. Comparative quantification of target genes expression in the samples was performed based on cycle threshold (Ct) normalized to housekeeping genes, using the ΔΔCt method (Livak and Schmittgen, 2001).

3.5.5 Evaluation of proliferation and apoptosis of cardiac primitive cells

Normal and pathological heart-derived CD117-positive cells were starved in serum-free medium for 24h. For evaluation of proliferation, quiescent cells were then incubated with the complete medium or cardiac fibroblast-conditioned medium for 24h, and 5-bromo-2'-deoxyuridine (BrdU) 10μM was added for 1h. Incorporation of BrdU was evaluated using BrdU Labeling and Detection Kit (Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol. The cells were fixed in acidic ethanol and incubated with anti-BrdU monoclonal antibody in incubation buffer containing nucleases. After incubation with anti-mouse-Ig-fluorescein, the nuclei were counterstained with DAPI and the stained area of culture dish was mounted in Vectashield (Vector Labs, Burlingame, CA, USA).

For evaluation of apoptosis, cells were incubated with 3% hydrogen peroxide in the complete medium or cardiac fibroblast-conditioned medium for 24h and fixed in 1% paraformaldehyde. The fragmentation of DNA was detected using the ApopTag Plus Fluorescein In Situ Apoptosis Detection Kit (Merck Millipore) based on terminal transferase dUTP nick end labeling, according to the manufacturer's protocol. Nuclei were counterstained with DAPI (Merck Millipore) and the stained area of culture dish was mounted in Vectashield (Vector Labs, Burlingame). For evaluation of apoptosis of cells seeded on polyurethane scaffold, cells were stained only with DAPI and the signs of the apoptotis, such as nuclear condensation and apoptotic bodies formation was looked for on microscopic observation.

Microscopic analysis was performed with a Leica DMLB microscope equipped with epifluorescence EL6000 system (Leica Microsystems, Wetzlar, Germany). Pictures were taken with digital camera connected to the microscope (Leica DFC345FX) and then merged with the software Leica Application Suite 3.6.

3.5.6. Evaluation of migration of cardiac primitive cells

For the evaluation of migration of cardiac primitive cells in the presence of fibroblast-derived cardiac matrix, cells were grown to confluence and a thin scratch was introduced on culture plates with a pipette tip, producing a cell-free zone (scratch-wound assay). The plates were observed at an inverted phase contrast microscope (Olympus Italia) and photographed with computer assisted digital camera (Soft Imaging System) at five distinct points, previously marked along the scratch on every culture plate, at various time points. Cell migration was quantified by measuring the width of the cell-free zone (distance between the edges of the scratched cell monolayer) at every time point with Cell A Imaging Software for Life Sciences Microscopy (Soft Imaging System).

3.6. Cardiac fibroblast-conditioned medium

3.6.1. Preparation

Conditioned medium was prepared by culturing cardiac fibroblasts in DMEM supplemented with 10% FBS in confluent state. The medium was collected every three days and diluted (1:3) with DMEM/F-12 (Sigma-Aldrich) supplemented as previously reported. Normal and pathological heart-derived cardiac primitive cells were starved in

serum-free medium for 24 h and then incubated with cardiac fibroblast-conditioned medium for 72h before proceeding to the evaluation of proliferation and apoptosis.

3.6.2. Characterization

The composition of cardiac fibroblast-conditioned medium was analyzed with RayBio® Human Cytokine Antibody Array. The array membranes with primary antibodies were blocked and incubated with undiluted conditioned medium obtained from culture of fibroblasts from normal or pathological heart. After washing, the membranes were incubated with a cocktail of biotin-conjugated antibodies, followed by incubation with HRP-conjugated streptavidin in blocking buffer. Signals were detected by chemiluminescence and autoradiography.

3.7. Cardiac primitive cell culture and evaluation on polyurethane scaffold

3.7. 1. Cardiac primitive cell seeding

Pathological heart-derived cardiac primitive cells were seeded onto each polyurethane scaffold (6mm diameter), previously sterilized by UV exposure for 20 min and placed in 96-well flat bottom cell culture plates. Cells were also seeded on 96-well conventional tissue culture plates as a control. Cells were cultured on control scaffolds, gelatine-coated scaffolds and laminin-1-functionalized scaffolds for 7, 14 and 21 days. The constructs were observed with an inverted phase contrast microscope (CKX41; Olympus Italia) equipped with a digital camera (Color View IIIu Soft Imaging System).

3.7.2. Embedding and sectioning of cell-scaffold constructs

Constructs were fixed in 4% paraformaldehyde for 20 minutes at room temperature. Next, scaffolds were incubated with 30% sucrose at 4^{o}C overnight and embedded in tissue freezing medium (TFM, Leica Biosystems) in disposable moulds specifically designed for cryosectioning (Bio-optica). To ensure quick freezing the disposable moulds were immersed in liquid nitrogen until TFM turned white. Frozen specimens were stored at -80^{o}C until cryosectioning. Slices of 10μm were cut in the cryostat at −20°C and cryosections were placed onto a microscope slide. Sections were stained as described in section 3.5.1.

3.8. Statistical analysis

All numerical data are presented as mean±SEM. Statistical differences between groups were evaluated with Student's two-tailed unpaired t-test; $p< 0.05$ was considered significant.

4. Results

4.1. Culture of cardiac primitive cells in standard conditions

4.1.1. Characterization of CPC population

CD117-positive cells were isolated from primary cardiac cell culture by immunomagnetic cell sorting (as described in Materials and Methods section 3.2). Purity of sorted cells was determined by immunofluorescence and reached 98%. The culture consisted of CD117-positive cardiac lineage-negative cells and CD117-positive cells that expressed markers of commitment towards cardiac cell lineages. Fluorescent immunolabelling revealed the presence of progenitors and precursors of three cardiac cell lineages (Fig.1): cardiomyocyte (with progenitors expressing transcription factor Nkx2.5 and precursors expressing α-sarcomeric actin in the cytoplasm), endothelial (with progenitors expressing transcription factor Ets-1 in the nuclei and precursors expressing also FVIII in the cytoplasm), and smooth muscle cells (with progenitors expressing transcription factor GATA6 in the nuclei and precursors expressing also smooth muscle actin in the cytoplasm).

4.1.2. Commitment and maturation of CPCs from normal and pathological hearts

The commitment and maturation of cardiac CD117-positive CPCs was examined at protein and mRNA level. The population of CD117-positive cells from adult human heart consisted of CPCs at different stages of their differentiation. The expression of the transcription factor Nkx2.5 identified putative cardiomyocyte progenitors. Cardiomyocyte precursors differed from progenitors by the expression of cytoplasmic proteins, such as α-sarcomeric

actin and cardiac myosin light chain. By way of analogy, endothelial and smooth muscle cell progenitors were identified by the expression of transcription factor Ets-1 and GATA6, respectively, while the precursors of the respective cell lineages expressed also factor VIII or smooth muscle actin in the cytoplasm. Consequently, we identified progenitors and precursors of cardiomyocytes, endothelial and smooth muscle cells in both CPC-N and CPC-P *in vitro* (Fig.1). Quantification of cells of different cardiac cell lineages (expressed as a percentage of positive cells in each maturation stage for every lineage among all CD117-positive cells in culture), based on the immunofluorescent staining of the abovementioned specific markers, revealed significant differences between the normal and pathological cell populations. In particular, the population of CD117-positive CPC-N was composed of twice as many precursors as progenitors (11.57 ±2.28 % and 22.58±0.87 %, respectively; n=3, $p<0.05$). On the contrary, the progenitors were about three times more numerous than precursors among CPC-P (46.82±10.42 % and 16.79±2.42 %, respectively; n=3, $p<0.05$). Of note, the proportion of committed cells (i.e., cells expressing at least one of the nuclear or cytoplasmic markers of differentiation toward one of the cardiac cell lineages) among the CD117-positive cells increased 1.86-fold ($p<0.05$) in the pathological conditions (Fig.2). Accordingly, compared with CPC-N, there was a higher expression of Nkx2.5, α-sarcomeric actin, and myosin light chain; VEGFR-2 and factor VIII; GATA6 and smooth muscle actin in the CPC-P (Fig.3).

Gene expression in cardiac CD117-positive CPC-N and CPC-P was examined by stem cell-specific PCR-based microarray employing 942 genes relevant for human stem cells and their differentiation; 936 genes passed the quality control, i.e., the spots were free of

hybridization artefacts and their signal intensity was satisfactory. We confined our analysis to the genes with at least 1.7-fold differential expression and we identified alterations in the expression of 77 genes, of which 38 were transcriptionally downregulated (Tab.1) and 39 were upregulated (Tab.2) in CPCs from hearts with ischemic cardiomyopathy (i.e. CPC-P). The classification based on the gene functional annotation returned developmental process as the top category for downregulated genes (Fig.4a). Additionally, these genes were annotated in the following groups: nervous system development and neurogenesis, skeletal development, bone and cartilage development. The downregulated genes included snail homolog 1 (SNAI1), jagged 1 (JAG1), ephrin-B1 (EFNB1), all involved in the early stages of developmental processes, and Mdm2, p15, p16, clustered in cell cycle control functional group. Genes upregulated in CPC-P belonged to the groups termed organ development and cell differentiation (in particular, mesenchymal cell differentiation and heart development), as well as response to wounding, external stimuli, and stress (Fig.4b). Interestingly, the upregulated transforming growth factor β3 (TGFβ3), hepatocyte growth factor (HGF), endothelin receptor type B (EDNRB), and mesoderm-specific transcript (MEST) belonged to epithelial–mesenchymal transition and mesenchymal cell differentiation functional groups. The results of microarray for the selected genes of interest were validated and confirmed by real time RT-PCR.

Since the microarray analysis revealed the upregulation of genes involved in TGFβ receptor signalling pathway and its regulation in the adult human hearts with ischemic cardiomyopathy, we next focused on TGFβ-mediated signal transduction and examined the expression of TGFβ/BMP signaling pathway-related genes in PCR-based array (Fig.5). In

CPCs from pathological heart, the mRNA of TGFβ1 was upregulated with respect to normal CPCs, together with the transcription factors ID1 and c-Myc, while that of activin A receptor type II-like 1 (ACVRL1) was downregulated. Interestingly, the transcriptional expression of interleukin 6 (IL6) and integrin β5 (ITGB5) increased, while that of integrin β7 (ITGB7) decreased significantly in the pathological conditions.

4.1.3. Proliferation and apoptosis of CPCs from normal and pathological hearts

Proliferation of CD117-positive CPCs was assessed by the BrdU incorporation in the S-phase of cell cycle (Fig.6a). Among the CPCs isolated from adult human hearts with ischemic cardiomyopathy (i.e. CPC-P), the proportion of proliferating cells reached 29.08±3.12 % and was 2.15 times higher compared to that of the normal CPCs (13.55±1.17 %, n=3, $p<0.05$). However, the CPC-P were 2.48 times more susceptible to apoptosis (Fig.6b): oxidative stress *in vitro* induced apoptosis in 1.7±0.3 % of CPC-N and in 4.21±0.66 % of CPC-P (n=3, $p<0.05$).

Since the proper balance between symmetric and asymmetric stem cell division is crucial for both stem cell population maintenance and tissue homeostasis, we have identified actively cycling cells expressing Ki67 and analyzed Notch distribution in cells undergoing cytokinesis (Fig.7). In the CPC-N, symmetric and asymmetric divisions were equally frequent, whereas in CPC-P the symmetric to asymmetric division ratio was almost 1:2 (n=3, $p<0.05$).

4.2. Culture of cardiac primitive cells in the presence of cardiac fibroblast-derived matrix

4.2.1. Cardiac matrix composition

Fibroblasts isolated from samples of adult human heart were cultured in confluent state allowing for cardiac extracellular matrix deposition *in vitro* (Fig.8). Incubation with basic solution of 0,5 M NaCl and 10mM Tris-base to extract soluble proteins, followed by incubation with 1% SDS induced membrane permeabilization and cell lysis, resulting in the removal of fibroblasts. Denudated matrix adhered to culture plate; its presence was observed at phase contrast microscope (Fig.9a), while its composition was revealed by indirect immunofluorescent staining of representative extracellular matrix glycoproteins and fibrillar proteins (Fig.9b): laminin, tenascin, fibronectin, and collagen.

Analysis of biomatrix composition by electrophoresis and immunoblotting revealed the differences in the composition of cardiac matrix produced by fibroblasts derived from normal (Cm-N) and pathological hearts (Cm-P). While Cm-N consisted mainly of collagen I and laminin-2, Cm-P was characterized by a higher content in tenascin-X and laminin-1 (Fig.10).

In the living tissue, the amorphous component of the extracellular matrix (so called interstitial fluid) is a site of deposition, diffusion and storage of numerous growth factors and cytokines secreted by local cells. This environment is substituted by culture medium *in vitro* and it is in the culture medium that the fibroblasts secrete soluble extracellular matrix components. Hence, the composition of culture medium conditioned *in vitro* by fibroblasts derived from normal (CF-N) and pathological (CF-P) hearts was evaluated by human cytokine antibody array. Comparison of the intensities of signals (Fig.11) quantified by

densitometry reflected the relative expression levels of cytokines secreted by CF-N and CF-P in the culture medium (Tab.3). Among the identified cytokines, those that reached higher concentration in the medium conditioned by CF-P with respect to CF-N were RANTES (23.7-fold), MCP-3 (21-fold), IGFBP1 (23.2-fold), EGF (1.8-fold), and Il-6 (2.3-fold difference, all n=3 in duplicate, $p<0.05$), while the concentration of others was higher in the medium conditioned by CF-N: SCF (1.5-fold), TGF-β3 (2.7-fold), BMP4 (4.4-fold difference, all n=3 in duplicate, $p<0.05$).

4.2.2. Biological characteristics of CPCs in the presence of normal and pathological cardiac matrix

Our model of cell culture *in vitro* allowed us to study the synthesis and secretion activity of cardiac fibroblasts isolated from normal or pathological adult human heart. The soluble factors synthesized by fibroblasts conditioned the medium used for fibroblast maintenance, while the fibrillar and non-fibrillar proteins were deposited as cardiac matrix on culture plates. Both extracellular matrix components were further analyzed by immunochemical methods (see above), and their biological function was tested on CPCs cultured in the presence of cardiac fibroblast-deposited extracellular matrix or cardiac fibroblast-conditioned medium.

First, fibroblast-derived cardiac matrix was used as a substrate for the culture of CPCs *in vitro*. Given the differences in cardiac matrix composition, revealed earlier by immunoblotting, two types of cardiac matrix, i.e. Cm-N, produced by fibroblasts isolated from the fragments of adult human normal heart, and Cm-P, produced by fibroblasts

isolated from the fragments of adult human hearts with ischemic cardiopathy, were used in this part of the study, enabling us to compare the effects of cardiac extracellular matrix typical of normal and pathological conditions on CPC proliferation, apoptosis, and migration *in vitro*.

Proliferation of CPC-P on Cm-P was 1,4-fold higher with respect to control (n=3, $p<0.05$) and 1,7-fold higher with respect to Cm-N (n=3, $p<0.05$). Although the percentage of proliferating cells was always significantly lower in the respective population of CPC-N, similar trend was noted when these cells were cultured on Cm-N and Cm-P (Fig.12a). The presence of fibroblast-derived cardiac matrix protected both populations of CPCs from apoptosis provoked by oxidative stress (n=6, $p<0.05$ with respect to control), although no statistically significant advantage of specific cardiac matrix type was evident (Fig.12b). Only in CPC-P an influence of Cm on migration speed could be observed, with its highest value on Cm-N; in the presence of Cm-P, it was similar to that of control but significantly slower when compared with the speed of migration on Cm-N (n=9, $p<0.05$) (Fig.12c).

The effects of extracellular matrix on cell maturation were studied by real time RT-PCR on CPCs cultured in the presence of extracellular matrix typical of normal and pathological heart for 14 days. Of note, the expression of several markers of cardiomyocyte (NKX2.5, MYH6), endothelial (ETS1, FVIII) and smooth muscle (GATA6, SMA) cell lineage at baseline in the population of CPC-P was significantly higher with respect to CPC-N (Fig.13). In the presence of cardiac matrix typical of pathological heart the expression of Nkx2.5 increased in CPC-N (with the effect of Cm-N being higher than that of Cm-P), but

not in CPC-P. For the endothelial cell lineage, the expression of transcription factor ETS1 increased, but at the same time that of cytoplasmic FVIII decreased moderately in CPC-P in the presence of Cm-P. While the expression of both those endothelial markers was much lower in CPC-N at baseline, in the presence of Cm-P their expression increased significantly. Similar differences between CPC-N and CPC-P regarded also the expression of smooth muscle cell precursor and progenitor markers (GATA6 and SMA). The presence of cardiac extracellular matrix *in vitro*, independently of its type, increased the expression of GATA 6 and SMA in CPC-N, while in CPC-P the maturation state of smooth muscle primitive cells was influenced by the type of cardiac matrix. In particular, an increase in the expression of GATA6 was noted in the presence of Cm-N, while SMA gene expression reached the highest value in the presence of Cm-P (Fig.14).

Second, we examined the effects of fibroblast-conditioned medium on CPC proliferation and apoptosis. As for the previous experiment, the observation that both the composition of culture medium conditioned by fibroblasts and the characteristics of CPCs were significantly different in the normal and pathological conditions was taken into consideration, hence four groups of culture plates were prepared and tested. The proportion of control CPC-N incorporating BrdU was not statistically different from that of CPC-N in the presence of medium conditioned by CF-N, but increased 1.6-fold when these cells were incubated in the medium obtained from CF-P culture (n=3, $p<0.05$). Proliferation of CPC-P was not significantly influenced by the presence of either type of cardiac fibroblast-conditioned medium (Fig.15a). Apoptosis rate of CPC-N diminished 2-fold when these cells were cultured in the medium conditioned by CF-N, while the effect of CF-P was not

statistically significant(Fig.15b). By contrast, in the presence of medium conditioned by CF-P apoptosis of CPC-P diminished 3.5-fold with respect to control (n=3, $p<0.05$).

4.3. Culture of cardiac primitive cells on biomimetic scaffolds

4.3.1. Scaffold seeding

CPCs cultured in the presence of the scaffolds were observed using optical and confocal fluorescent microscopy; the latter was performed after labelling fixed cells with phalloidin and antibodies against Ki67. On the third day of culture, cells were found to adhere to scaffolds and cover the surface of the scaffold *trabeculae* spreading across the pores (Fig.16a). The proliferation-associated protein Ki67*,* which is expressed by cells in late G1, S, G2 and M phases, but not in resting cells in G0, was present in cell nuclei. Confocal image analysis revealed that the cells stretched out in three dimensions, between the trabeculae of the same and of the adjacent scaffold layers (Fig.16b).

4.3.2. Proliferation and apoptosis of CPCs on biomimetic scaffolds

Laminin-1 predominates among the laminin forms during early embryogenesis and further organogenesis. Its unique role is underlined by the fact that embryogenesis will not proceed in the absence of this form of laminin [86]. Moreover, it has been found to be re-expressed in heart regeneration following tissue damage [10]. With the aim to stimulate CPCs with the biomimetic properties of synthetic scaffold used for cell delivery, those scaffolds were functionalized with laminin-1 and their effects on proliferation and apoptosis of CPCs were compared with control scaffolds (not functionalized and functionalized with gelatin).

Proliferation of CPC-P cultured on polyurethane control, gelatine- and laminin-1-functionalized scaffolds were assessed at two time points by identifying actively cycling cells expressing Ki67 (Fig.17a). At all time points (7 and 14 days), the rate of Ki67-positive cells was highest in the presence of laminin-1-functionalized scaffolds. This difference was particularly evident at 7 days, when the proportion of cycling cells was 3.3-fold higher (18.75±3.89%) with respect to both non-coated (5.6±1.15%) and gelatin-coated (5.66±0.4%) polyurethane scaffolds (n=3, $p<0.05$). Similarly, apoptosis of CPC-P was 2.4-fold and 1.4-fold lower at 7 days in the presence of laminin-1-functionalized scaffolds then non-coated and gelatin-coated scaffolds, resepctively (n=3, $p<0.05$) (Fig.17b).

5. Discussion

5.1. Progenitors and precursors of cardiac cell lineages in normal and pathological heart

In any self-renewing organ, there is an established tissue maintenance program that is responsible for the continuity of organ structure and function throughout life: damaged or aged and apoptotic cells are substituted by the differentiating progenies of stem cells residing in organ-specific niches. While previous studies described an increase in CD117-positive CPCs number following cardiac tissue damage [10], the present study of chronological age-matched CPCs in the normal and pathological conditions revealed that CPCs from the adult human pathological heart are committed to cardiac tissue specific fate: on the one hand genes related to stemness and multipotentiality, and those involved in neurogenesis, osteogenesis, chondrogenesis, and skeletal muscle development were downregulated; on the other hand there was an upregulation of genes and proteins whose expression correlates with cardiac tissue-specific development, differentiation, and maturation. Moreover, the activation of TGFb/BMP signaling pathway may correspond to epithelial–mesenchymal transition and mesenchymal cell differentiation. This possibility is corroborated by two recent findings: first, the induction of the epithelial–mesenchymal transition in epicardial cells from adult human heart gave rise to CD117-positive cardiac cells in vitro [44]; second, epicardially-derived cells can give origin to endothelial, smooth muscle [87], and arguably, cardiomyocyte precursors *in vivo* [88]. Despite the evident commitment of numerous cardiac progenitors, the number of more mature CPCs, i.e. cardiac lineage precursors was relatively low in ischemic heart [89]. Two possible

explanations offer themselves, namely that the differentiation program is inhibited and the early committed cells are destined to apoptosis in the pathological conditions or, alternatively, the cells continue on their route to terminal differentiation and maturation, which is associated with a loss of CD117 antigen expression. The results of the present study support the former; in fact, the rate of apoptosis among CPC-P was significantly higher with respect to CPC-N and the same was also true in the CD117-negative fraction of CPCs (data not shown). The extent of stem cell activation in pathological heart injury, when massive cardiac cell necrosis and apoptosis ensue, is presumably much higher than that providing for a physiological "wear and tear" repair. As such, cardiac stem cell activation in ischemic heart can be analogous to that occurring during organ development, when a formation of numerous fast-cycling transient amplifying primitive cells occurs and proliferation and apoptosis of the progenitors and precursors of different cell lineages are strictly regulated and coordinated in order to form a functionally competent tissue [90]. The results of the present study suggest that stem cell-mediated cardiac regeneration can be governed by the same principle: in pathological conditions, cardiac stem cells undergo asymmetric division (which was found to be the prevalent form of division of CPC-P) and give origin to numerous fast-cycling progenitors of cardiac cell lineages; apoptosis of these progenies can be imposed by the developmental program activated in cardiac tissue as a result of damage. In fact, the disruption of this program and, in particular, the inadequate incidence of apoptosis, can be responsible for cardiac malformation and impede the remodelling of developing tissue [91]. Alternatively, the incapacity of CPCs from pathological hearts to reach terminal differentiation can depend on the adverse effects of the microenvironment and the extrinsic stimuli that the cells endure in the pathological

conditions, resulting from changes in left ventricle loading conditions, neurohormonal activation, and alterations in tissue perfusion and metabolism, which affect repair and remodelling of the myocardium as a whole [92]. Another study that compared biological characteristics of cardiac stem cells from normal and pathological adult heart [93] pointed out that telomere shortening was a major trigger of cellular senescence and stem cell pool functional exhaustion. As a matter of fact, it was already reported that stem cell characteristics, including the expression and function of telomerase, were critically influenced by extrinsic signals derived from their environment [94]. Moreover, while the multipotent stem cells express telomerase, the CPCs are no longer able to preserve their telomeric DNA; hence the balance between proliferation and differentiation has to be tightly controlled in order to allow these cells to contribute to cardiac regeneration and to restore cardiac structure and function.

Extracellular matrix composition and structure, as well as a myriad of growth factors and cytokines that are stored and selectively or sequentially activated as a result of interactions with matrix components, all contribute to the maintenance of stem cell pool and to the commitment and terminal differentiation of stem cell progenies. The biological characteristics of the microenvironment change profoundly in the ischemic heart disease [95], influencing and modifying the characteristics of cardiac stem cells and CPCs. An interesting example of the extracellular matrix role in stem cell biology is the expression of integrins, transmembrane cell adhesion receptors that bind to extracellular matrix ligands, cell surface ligands, and soluble ligands to mediate the outside–inside signaling. From the analysis of PCR-based array in our study, it is apparent that the expression of integrin β7

(ITGβ7) is downregulated in CPCs in the pathological conditions. The effects of such a downregulation were recently revisited in hematopoietic stem cells [96]. ITGβ7 mutant mice displayed a transient increase of colony-forming unit progenitors in the bone marrow coupled with a proportional increase in the number of precursor cells in the peripheral blood. However, following tissue damage induced by exposition to phenylhydrazine, a number of splenic erythroid colony-forming units decreased with respect to control and anemia ensued. By way of analogy, cardiac stem cells in the pathological conditions could give rise to fast-cycling transient amplifying progenitors of cardiac cell lineages, which subsequently increase in number. Still, as indicated by the results of the present study, it appears that these cells fail to accomplish differentiation process, do not acquire functional competence and, as obsolete, are removed by apoptosis.

In conclusion, a series of biological events seems responsible for the failure of CPCs to reach terminal differentiation and functional competence in pathological hearts. From the foregoing discussion, it would seem that both intrinsic tissue regeneration program and microenvironmental cues regulate the proliferation/differentiation balance of CPCs. The results of our study indicate that this balance is disturbed in chronic ischemic heart disease. Hence, our next efforts were aimed at studies of the effects of microenvironmental changes on CPCs.

5.2. Cardiac matrix in the normal and pathological heart and its effects on cardiac primitive cells

An organized extracellular matrix is necessary for the arrangement of cells and thus for the maintenance of structure in any given tissue; this intricate interlocking mesh of fibrillar and nonfibrillar proteins and glycosaminoglycans also determines tissue biomechanical properties. Numerous cell types are able to synthesise and secrete extracellular matrix components, and their activity is regulated and changes in response to various stimuli, such as inflammation, biomechanical stress, and tumorigenesis [97], while already deposited extracellular matrix proteins are targeted by specific enzymes, metalloproteinases, that are responsible for the continuous remodelling of matrix and allow cell movement and size adaptation. In any necrotic tissue, fibronectin and collagen deposition is responsible for scar formation, which preserves wall integrity and thickness, but can be deleterious for tissue and cell function. Also in the ischemic heart disease or cardiac pressure overload, cardiomyocyte hypertrophy is accompanied by interstitial fibrosis, while the necrotic tissue is substituted by a scar. However, in the chronic conditions such remodelling increases wall stiffness, contributes to cardiomyocyte slippage, and worsens the contractile properties of the myocardium [24]. On these bases, it is evident that secretory activity of fibroblasts, which exceed in number any other cell type in the myocardium, influences myocardial function: it is determinant in normal, but it can be detrimental in pathological conditions. Hence, the synthetic and secretory activity of cardiac fibroblasts isolated from normal or pathological adult human heart was studied in the present model of fibroblast cell culture *in vitro*. The soluble factors conditioned the medium used for fibroblast maintenance, while the fibrillar and nonfibrillar proteins were deposited as cardiac matrix on culture plates. Both extracellular matrix components were analyzed by immunochemical methods, while their biological function was tested on CPCs *in vitro*.

Several authors have described the changes of cardiac stem/primitive cells biological properties in pathological conditions or aging [93,89], but the contribution of microenvironment has not been considered and sufficiently acknowledged due to the lack of an appropriate model for cell-matrix interaction studies. The molecular constituents of extracellular matrix play major role in the responses of cells to their local microenvironment. Both direct stimulation of the specific receptors by growth factors stored in extracellular matrix and indirect activation by specific integrin expression and clustering can transmit extracellular biochemical inputs along the intracellular signaling pathways that regulate cell proliferation, survival, and migration. As a matter of fact, the expression of integrin subunits in the same cardiac primitive cell population changes in qualitative and quantitative manner depending on cell culture substrate (Cm-N or Cm-P, data not shown). From the above observations it follows that the composition of extracellular matrix must be taken into consideration when planning cardiac regeneration based on stem/primitive cell transplantation or activation.

In mammals, nearly 300 proteins (among which collagen subunits, proteoglycans, and glycoproteins) have been identified as components of extracellular matrix [98]. So far, only few of them have been recombinantly expressed or purified and are available for the studies of their role in cell biology *in vitro*. Although the extracellular matrix of each tissue contains similar components, the combination of proteins and proteoglycans is tissue-specific [99], therefore it is arguable that only cardiac extracellular matrix should provide the proper biological and chemical cues for myocardial regeneration. Synthesis and

deposition of extracellular matrix in the myocardium occurs principally by cardiac fibroblasts and is influenced by neurohumoral activation and bioactive factors. Moreover, cardiac fibroblasts are able to sense mechanical stresses and signals from the surrounding extracellular matrix via integrins and may modify their secretory activity accordingly [100], influencing other interstitial and parenchymal cells [101]. In the present study, the effects of both amorphous and fibrillar components typical of cardiac microenvironment were characterized and their influence on CPC was studied *in vitro*. It is possible to envisage that both types of CPCs, normal and pathological, could be used for myocardial regeneration in different biological and clinical settings. The results of the present study indicate that in order to augment the proliferation of CPC-N *in vitro* or *in vivo*, one should consider the use of matrix components resulting from activity of fibroblasts already stimulated by the pathological conditions, while the protection of CPC-N from apoptosis could be accomplished by the components of matrix typical or particularly present in the normal heart. If CPC-P were to be used in cell therapy, the cardiac matrix already present in pathological conditions could protect these cells from apoptosis, however, the proliferation of these cells cannot be influenced (further increased) by cardiac fibroblast-derived matrix components. Interestingly, migration of CPC-N was not significantly influenced by matrix type (normal or pathological), while that of CPC-P increased only on matrix deposited by fibroblasts typical of normal heart. Considering the variations and interactions among extracellular matrix constituents, the possible influence on cardiac primitive cells is difficult to predict. Hence, the model of extracellular matrix production by cardiac fibroblasts and its use as a substrate for cardiac primitive cell culture described in the present study may fill this gap and improve the results of cell transplantation.

5.3. Biomimetic scaffolds seeded with cardiac primitive cells

Multicellular structures rely on the presence of extracellular matrix to provide scaffolding for cells and tissue compartments. The extracellular matrix structure has implications for both the cellular activity and mechanical performance of the tissue. The goal of tissue engineering is to develop constructs that are functionally equivalent to lost or damaged tissues. Hence, tissue engineering strategies are generally aimed at tissue regeneration mediated by purposely designed three-dimensional matrices (scaffolds) regulating cell function. This approach at tissue regeneration requires the design of appropriate scaffolding biomaterials with suitable three-dimensional architecture as well as the presence of molecules on the scaffold/biomaterial able to interact with cells via outside-inside signalling. So far, blends between synthetic polymers, such as polyurethane and polycaprolactone and natural proteins, such as gelatin, collagen and chitosan, were reported for the preparation of porous scaffolds for cardiac tissue engineering [102]. Segmented polyurethanes are particularly attractive, being elastomeric biomaterials with tunable mechanical properties, processability and good biocompatibility [103].

As previously reported, laminin-1 is the first glycoprotein expressed during early embryogenesis [104] and it is re-expressed in adult heart regeneration following tissue damage [10]. For this reason, its properties as a coating of polyurethane scaffold seeded with CPCs were tested in the present study. Indeed, laminin-1 coating increased the proliferation and survival rate of CPCs with respect to polyurethane scaffold without

coating or coated with gelatin. In the light of the results of the present study, the approaches based on the modification of biomaterials with bioactive molecules (such as native long chain proteins or short peptide sequences derived from intact extracellular matrix, which can incur specific interactions with cell receptors) should benefit from the description of biological characteristics of cells in the presence of naturally-derived cardiac matrix mimicking myocardial microenvironment.

6. Conclusions

The results of the study highlighted the role of microenvironment in cardiac regeneration. In the era of stem cell based tissue regeneration and tissue engineering, the choice of natural biological components and biomimetic materials used for myocardial regeneration should be based on their specific role on cardiac cell biology. Moreover, changes that take place in chronic pathological conditions should be taken into consideration when planning stem cell-based therapy. This study indicated the potential use of cardiac fibroblast-derived matrix for *in vitro* studies of the interactions between components of extracellular matrix and cardiac primitive cells responsible for cardiac self-renewal. Biochemical stimulation of CPCs by extracellular matrix fibrillar and amorphous components can be applied to study how the combination of these factors lead to changes in CPC phenotype and function in normal and pathological conditions. The goal of regenerative medicine and tissue engineering to regenerate damaged myocardium can be achieved by further investigations into cell-matrix interactions at different stages of cardiac ischemic disease progression.

The extent to which the composition of the biological matrix substrate or scaffold coating can be modified in order to promote cell growth, survival and differentiation remains controversial. Although we are constantly aiming at nature mimicking in organ regeneration, this goal is still far from being reached. It can be argued that in this particular field, progress can be achieved by aiming high but not over demanding. Step by step, ensuing knowledge should deepen our understanding of the biochemical cues that regulate

cardiac primitive cell survival, proliferation, migration, and differentiation, and as a result guide cardiac tissue regeneration.

FIGURES AND TABLES

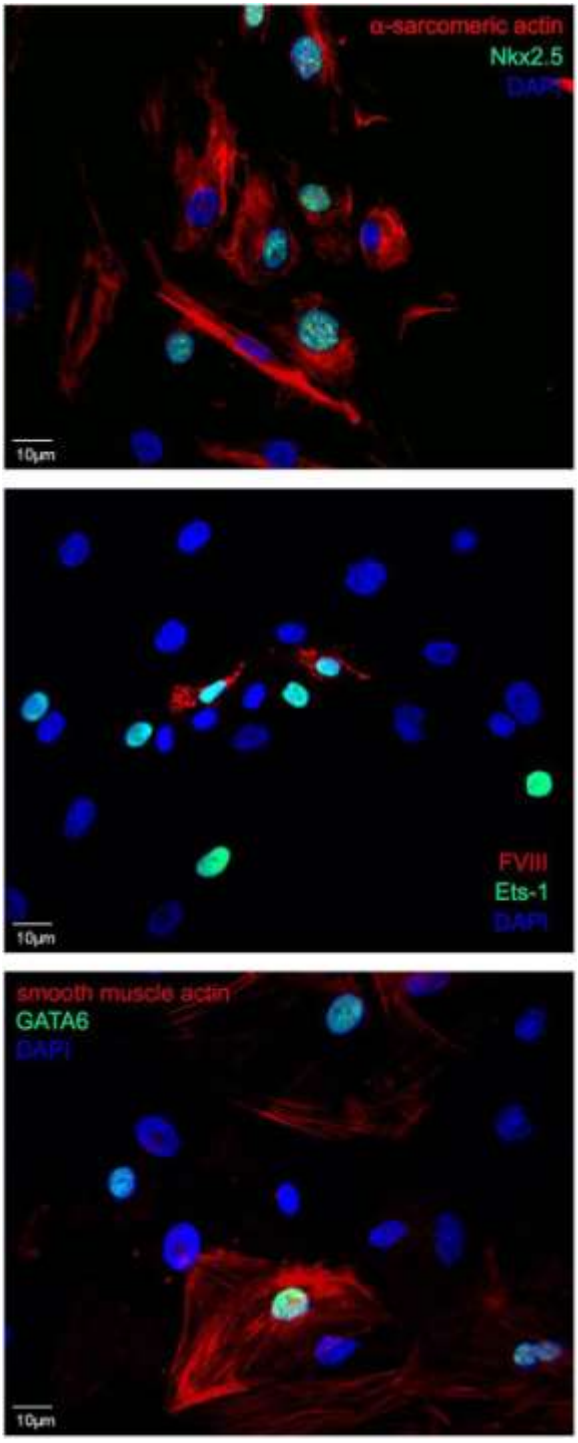

Figure 1. Immunofluorescent staining of cardiac CD117-positive cell population *in vitro*, indicating the presence of cardiomyocyte, endothelial and smooth muscle cells.

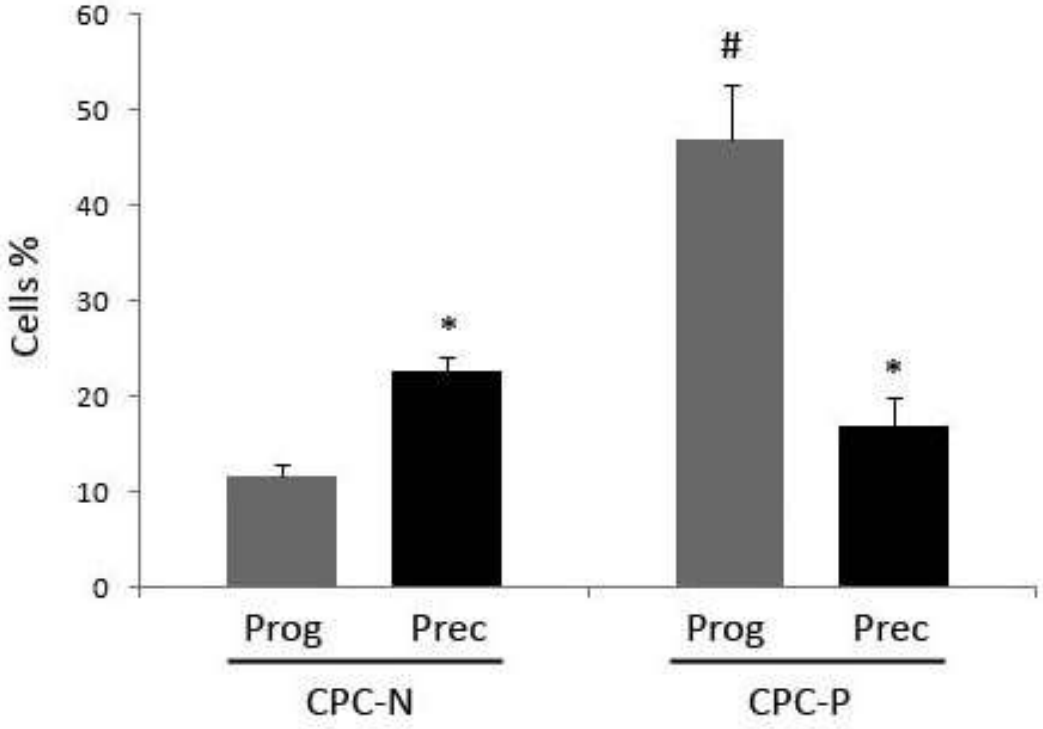

Figure 2. Quantification of progenitors (Prog) and precursors (Prec) of cardiac cell lineages in the population of cardiac primitive cells from normal (CPC-N) and pathological hearts (CPC-P). * $p<0,05$ vs Prog; # $p<0,05$ vs CPC-N.

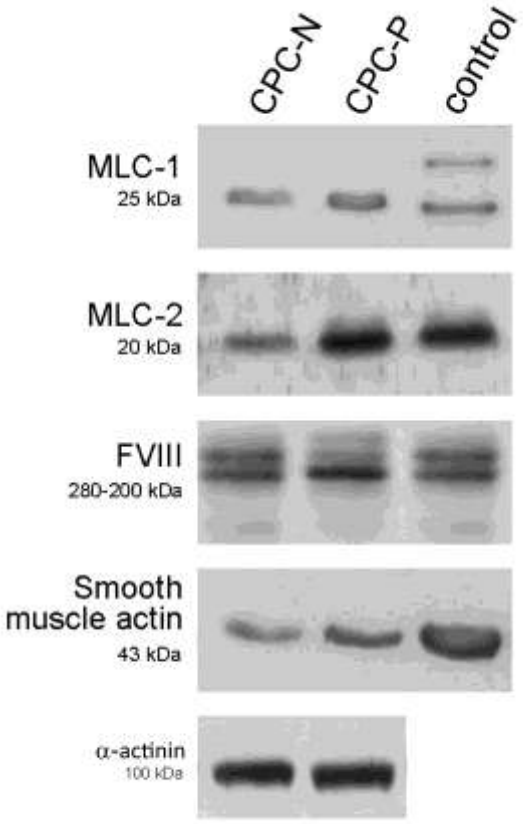

Figure 3. Expression of cardiac lineage specific proteins evaluated by electrophoresis and immunoblotting in the population of cardiac primitive cells from normal (CPC-N) and pathological heart (CPC-P).

Symbol	Description	Fold change	CV (%)
CRIP1	CYSTEINE-RICH PROTEIN 1	0,20	5
GJA5	GAP JUNCTION ALPHA-5 PROTEIN (CX40).	0,21	8
GJA4	GAP JUNCTION ALPHA-4 PROTEIN (CX37).	0,23	5
TNFSF4	OX40 LIGAND	0,28	15
PRPH	PERIPHERIN	0,31	1
DPYSL3	DIHYDROPYRIMIDINASE RELATED PROTEIN-3	0,32	9
FABE	FATTY ACID-BINDING PROTEIN, EPIDERMAL	0,32	3
KCND2	VOLTAGE-GATED POTASSIUM CHANNEL	0,33	15
COL1A1	COLLAGEN ALPHA 1	0,34	12
SELL	L-SELECTIN PRECURSOR	0,38	6
LMO2	RHOMBOTIN-2	0,40	10
EFNB1	EPHRIN-B1 PRECURSOR	0,42	9
ANGPT2	ANGIOPOIETIN-2 PRECURSOR	0,42	11
CTF1	CARDIOTROPHIN-1	0,43	21
CRABP2	RETINOIC ACID-BINDING PROTEIN II	0,43	11
TUBB3	TUBULIN BETA-3 CHAIN	0,43	11
EDN1	ENDOTHELIN-1 PRECURSOR	0,45	9
SNAI1	ZINC FINGER PROTEIN SNAI1	0,46	10
MDM2	UBIQUITIN-PROTEIN LIGASE E3 MDM2	0,47	7
VIM	VIMENTIN	0,48	7
SMURF1	SMAD UBIQUITINATION REGULATORY FACTOR 1	0,49	7
NR2F1	COUP TRANSCRIPTION FACTOR 1	0,49	6
PRRX1	PAIRED MESODERM HOMEOBOX PROTEIN 1	0,49	4
JAG1	JAGGED 1 PRECURSOR (NOTCH LIGAND JAGGED 1)	0,49	3
WNT10B	WNT-10B PROTEIN PRECURSOR	0,49	13
ALPL	ALKALINE PHOSPHATASE (LIVER/BONE/KIDNEY ISOZYME)	0,51	7
SERPINH1	SERPIN H1 PRECURSOR (COLLAGEN-BINDING PROTEIN)	0,51	5
CD24	SIGNAL TRANSDUCER CD24 PRECURSOR (NECTADRIN)	0,52	9
CDKN2B	CYCLIN-DEPENDENT KINASE 4 INHIBITOR B	0,52	9
CDKN2A	CYCLIN-DEPENDENT KINASE 4 INHIBITOR A	0,52	5
FLT1	VASCULAR ENDOTHELIAL GROWTH FACTOR RECEPTOR 1	0,52	3
NTRK1	HIGH AFFINITY NERVE GROWTH FACTOR RECEPTOR	0,52	10
SEMA3B	SEMAPHORIN 3B PRECURSOR	0,52	10
MMP14	MATRIX METALLOPROTEINASE-14 PRECURSOR	0,54	8
MC1R	HYPOTHETICAL PROTEIN (MGC16491).	0,57	15
TUBA	TUBULIN ALPHA-UBIQUITOUS CHAIN	0,58	7
SELE	E-SELECTIN PRECURSOR	0,58	7
AGRIN	AGRIN PRECURSOR.	0,58	11

Table 1. List of genes that were transcriptionally downregulated in the population of cardiac primitive cells from pathological heart with ischemic cardiopathy with respect to normal heart, as evaluated by PCR-based stem cell specific microarray.

Symbol	Description	Fold change	CV (%)
TREM1	TRIGGERING-RECEPTOR TREM1	8,37	65
CHI3L1	CHITINASE-3 LIKE PROTEIN 1 PRECURSOR	7,43	10
CLDN5	CLAUDIN-5	6,51	15
MME	NEPRILYSIN	4,75	3
CLDN1	CLAUDIN-1	4,08	28
HPD	4-HYDROXYPHENYLPYRUVATE DIOXYGENASE	3,43	14
PF4V1	PLATELET FACTOR 4 PRECURSOR (CXCL4)	3,37	6
IL6	INTERLEUKIN-6 PRECURSOR (IL-6)	3,33	9
EDNRB	ENDOTHELIN B RECEPTOR PRECURSOR	3,24	7
MGST1	GLUTATHIONE S-TRANSFERASE	2,92	5
BEX1	PROTEIN BEX1 (BRAIN-EXPRESSED X-LINKED PROTEIN 1)	2,64	9
ITGA7	INTEGRIN ALPHA-7 CHAIN	2,63	5
POU2F2	OCTAMER-BINDING TRANSCRIPTION FACTOR 2 (OCT-2 FACTOR)	2,59	6
TNC	TENASCIN PRECURSOR	2,57	6
PGH2	PROSTAGLANDIN G/H SYNTHASE 2 PRECURSOR	2,57	8
IGFBP2	INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN 2 PRECURS.	2,47	13
MCAM	CD146 ANTIGEN PRECURSOR	2,30	13
TIMP1	METALLOPROTEINASE INHIBITOR 1 PRECURSOR	2,23	29
PRKCZ	PROTEIN KINASE C, ZETA TYPE	2,19	15
PON3	SERUM PARAOXONASE/ARYLESTERASE 3	2,17	15
HERMES	RNA-BINDING PROTEIN WITH MULTIPLE SPLICING (RBP-MS)	2,12	8
NGFB	BETA-NERVE GROWTH FACTOR PRECURSOR	2,04	7
FABP4	FATTY ACID-BINDING PROTEIN	2,03	10
COL7A1	COLLAGEN ALPHA 1(VII) CHAIN PRECURSOR	2,00	11
RAMP1	RECEPTOR ACTIVITY MODIFYING PROTEIN 1	2,00	8
BDNF	BRAIN-DERIVED NEUROTROPHIC FACTOR PRECURSOR	1,94	11
CITED2	CBP/P300-INTERACTING TRANSACTIVATOR 2	1,94	13
PEG1-MEST	PEG1/MEST PROTEIN	1,89	5
TGFB3	TRANSFORMING GROWTH FACTOR BETA 3 PRECURSOR	1,87	10
HGF	HEPATOCYTE GROWTH FACTOR PRECURSOR	1,85	12
HTR2B	5-HYDROXYTRYPTAMINE 2B RECEPTOR	1,75	8
ACTA2	AORTIC SMOOTH MUSCLE (ALPHA-ACTIN 2).	1,74	6
LIF	LEUKEMIA INHIBITORY FACTOR	1,74	6
CDO1	CYSTEINE DIOXYGENASE TYPE I	1,74	13
DAB1	DISABLED HOMOLOG 1	1,73	10
ACTG2	ACTIN, GAMMA-ENTERIC SMOOTH MUSCLE	1,72	9
PUMH1	HYPOTHETICAL PROTEIN KIAA0099 (PUMILIO 1)	1,71	14
ENG	ENDOGLIN PRECURSOR (CD105 ANTIGEN)	1,70	8

Table 2. List of genes that were transcriptionally upregulated in the population of cardiac primitive cells from pathological heart with ischemic cardiopathy with respect to normal heart, as evaluated by PCR-based stem cell specific microarray.

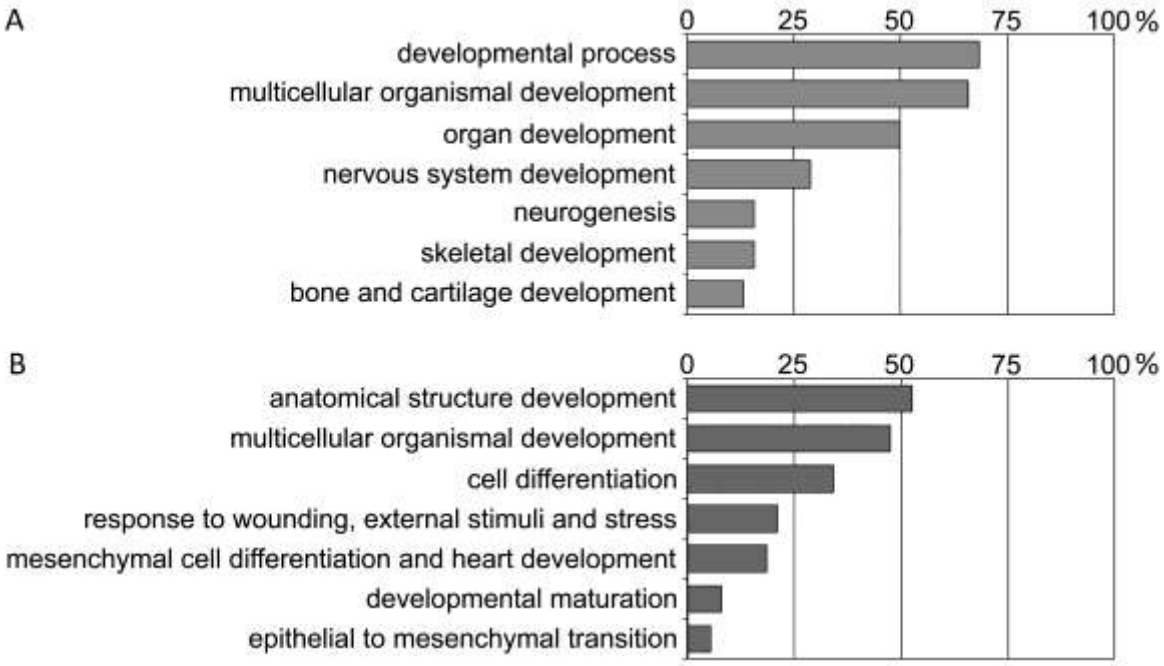

Figure 4. Functional classification of genes which expression was downregulated (A) and upregulated (B) in the population of cardiac primitive cells from pathological heart with ischemic cardiopathy with respect to normal heart, as evaluated by PCR-based stem cell specific microarray.

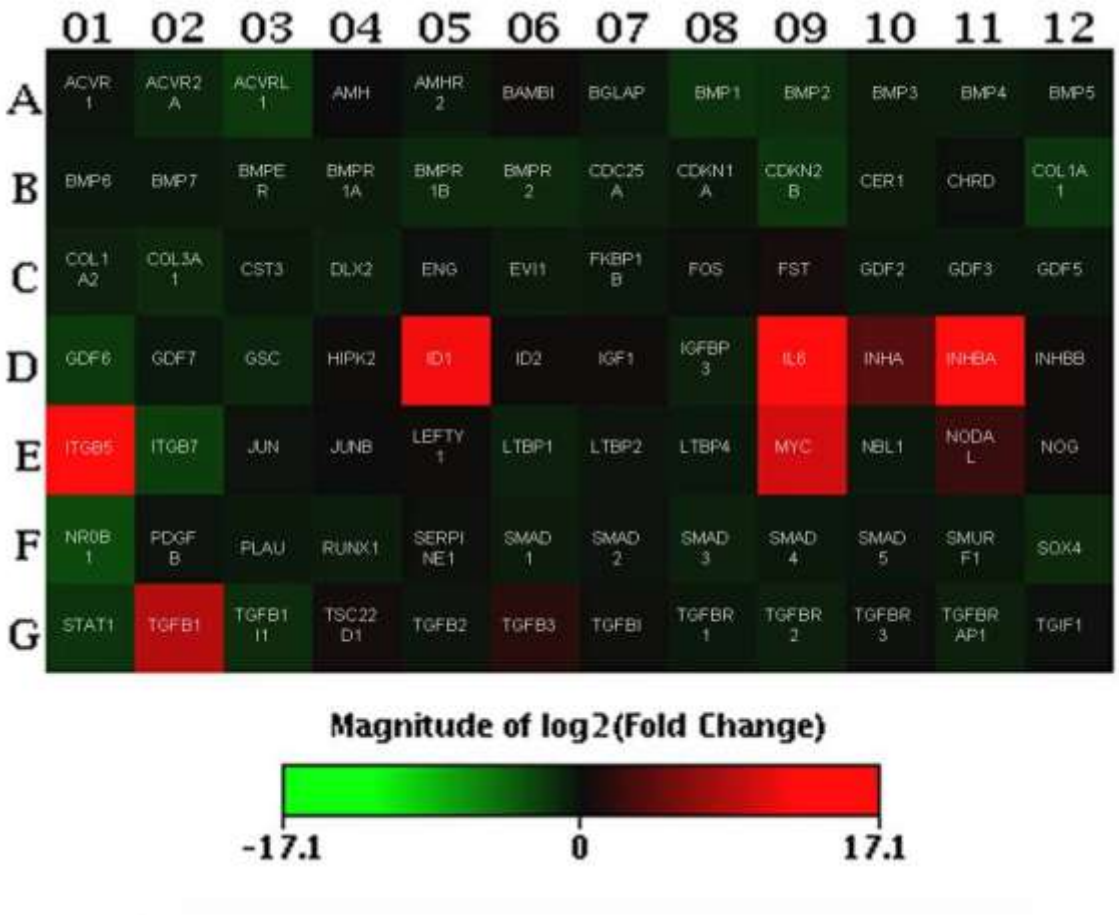

Figure 5. Changes in the expression of TGFβ/BMP signalling pathway related genes (red - upregulated, green - downregulated) in the population of cardiac primitive cells from pathological heart with ischemic cardiopathy with respect to normal heart, as evaluated by PCR-based array.

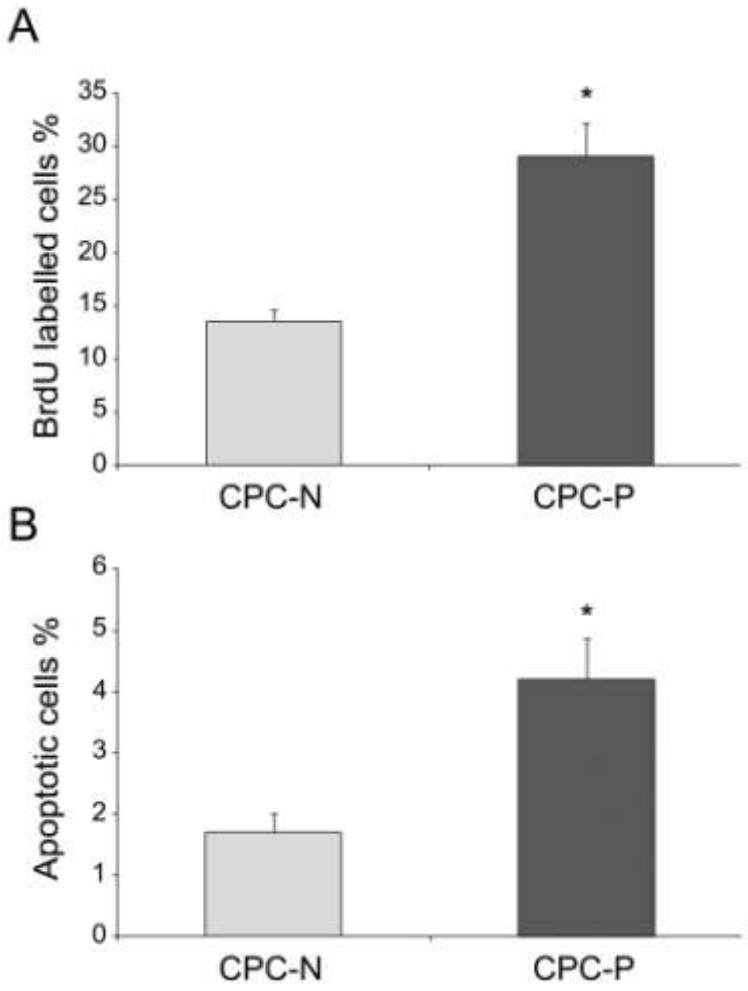

Figure 6. Proliferation (A) and apoptosis (B) of cardiac primitive cells from normal (CPC-N) and pathological heart (CPC-P) *in vitro*. * $p<0,05$ vs CPC-N.

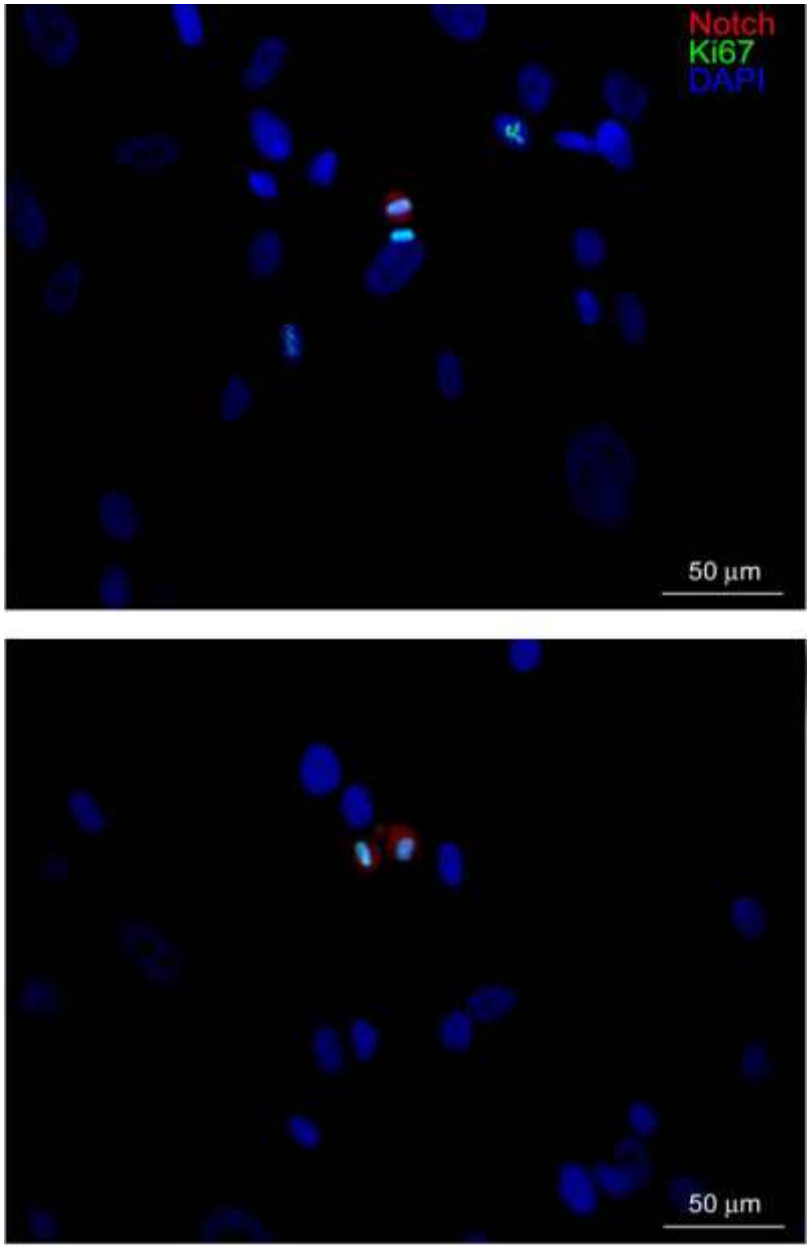

Figure 7. Representative images of asymmetric (upper) and symmetric (lower) division of cardiac primitive cells in vitro, as evidentiated by immunofluorescent staining of Ki67 and Notch.

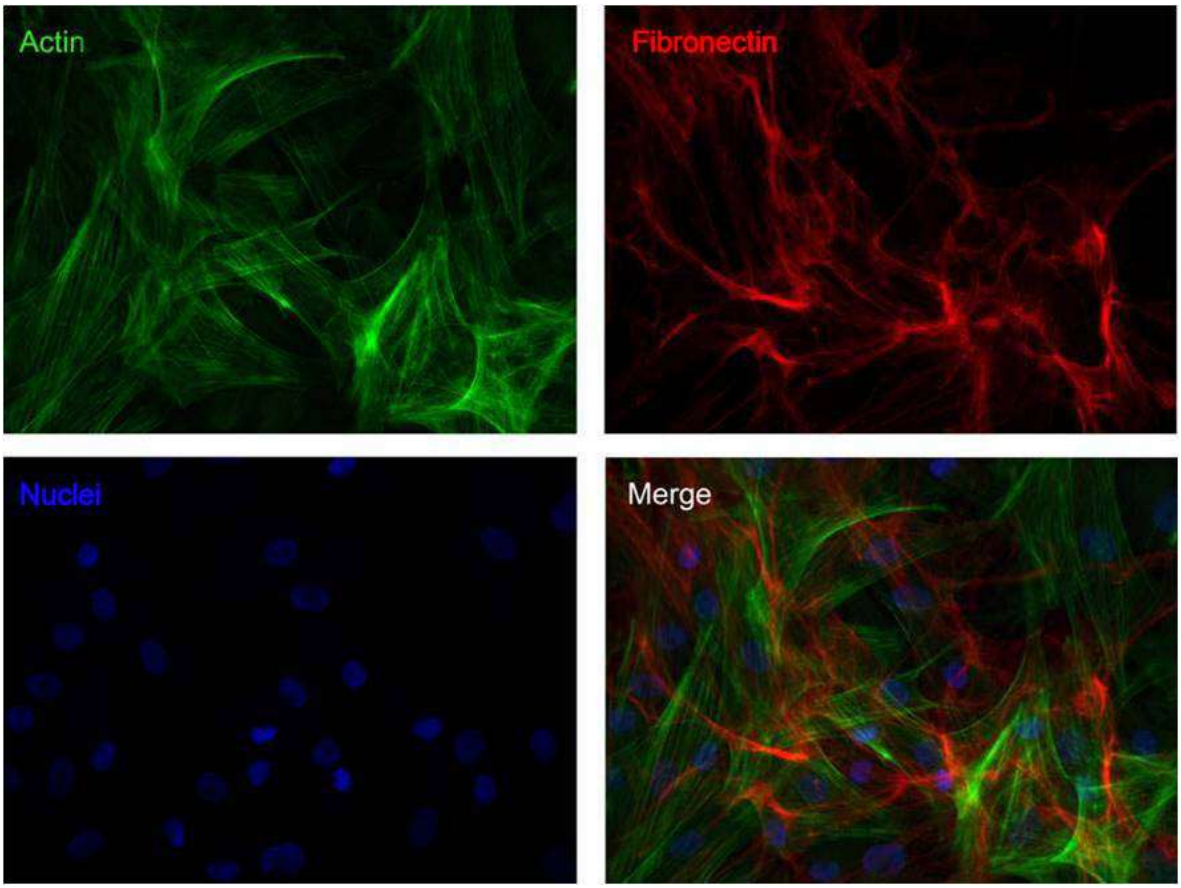

Figure 8. Immunofluorescent staining of cardiac fibroblast during matrix synthesis and deposition *in vitro*.

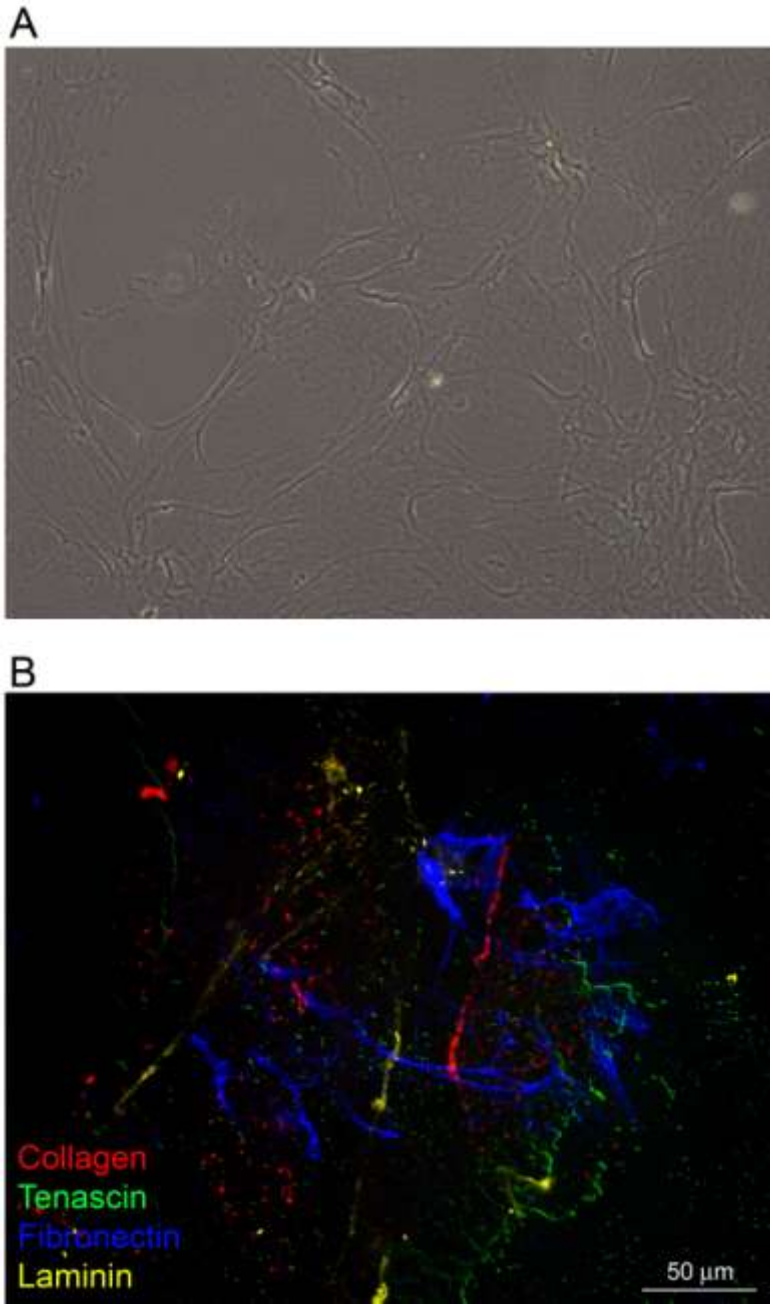

Figure 9. Cardiac matrix observed at phase contrast microscopy during fibroblast removal (A) and immunofluorescent staining of proteins typical of extracellular matrix deposited *in vitro* by cardiac fibroblasts (B).

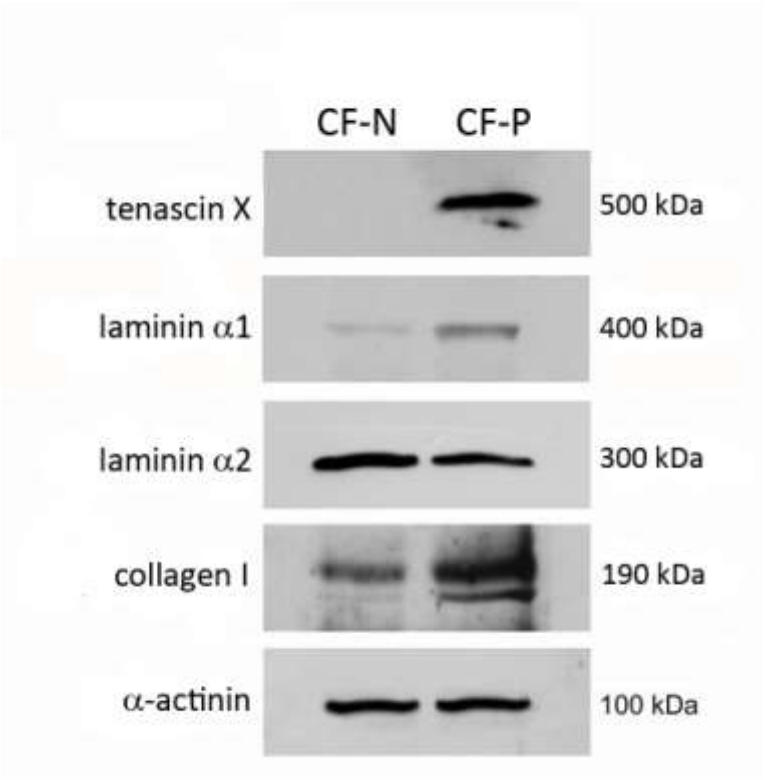

Figure 10. Expression of proteins typical of extracellular matrix deposited *in vitro* by cardiac fibroblasts from normal (CF-N) and pathological heart (CF-P).

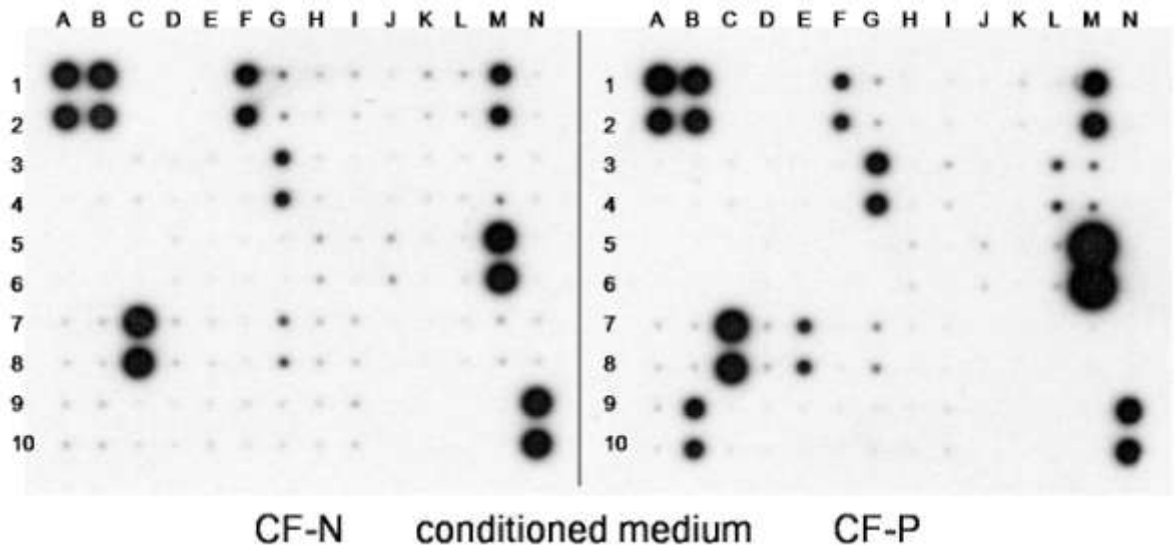

Figure 11. Membrane immunoabsorbent assay results indicating relative concentration of factors secreted in the medium (conditioned medium) by cardiac fibroblasts from normal (CF-N) and pathological (CF-P) heart.

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	CTR POS	CTR POS				ANGIOG ENIN	BDNF	BCL	BMP4	BMP6	CKβ8-1	CNTF	EGF	EOTAXIN
2	CTR POS	CTR POS				ANGIOG ENIN	BDNF 2,1-fold	BCL 3,7-fold	BMP4 4,4-fold	BMP6	CKβ8-1	CNTF	EGF 1,8-fold	EOTAXIN 2,4-fold
3			FGF-6				GCP-2	GDNF	GMCSF		IFN-γ	IGFBP1	IGFBP2	IGFBP4
4			FGF-6				GCP-2 2,5-fold	GDNF 3,4-fold	GMCSF 5,7-fold		IFN-γ	IGFBP1 23,2-fold	IGFBP2	IGFBP4 3,6-fold
5								IL-1ra		IL-3		IL-5	IL-6	
6								IL-1ra 2,3-fold		IL-3 2,2-fold		IL-5 1,7-fold	IL-6 2,3-fold	
7	LEPTIN	LIGHT	MCP-1	MCP-2	MCP-3		M-CSF	MDC	MIG				NT-3	PARC
8	LEPTIN	LIGHT	MCP-1	MCP-2 1,3-fold	MCP-3 21-fold		M-CSF	MDC	MIG 7,4-fold				NT-3	PARC 3,1-fold
9	PDGF BB	RANTES	SCF	SDF-1	TARC	TGF-β1	TGF-β3	TNF-α	TNF-β					CTR POS
10	PDGF BB	RANTES 23,7-fold	SCF 1,5-fold	SDF-1	TARC	TGF-β1 1,7-fold	TGF-β3 2,7-fold	TNF-α	TNF-β					CTR POS

Table 3. Changes in the concentration (red - decrease, green - increase) of factors secreted in the medium by cardiac fibroblasts from pathological heart with respect to normal heart, as evaluated by densitometric analysis of membrane immunoabsorbent assay results.

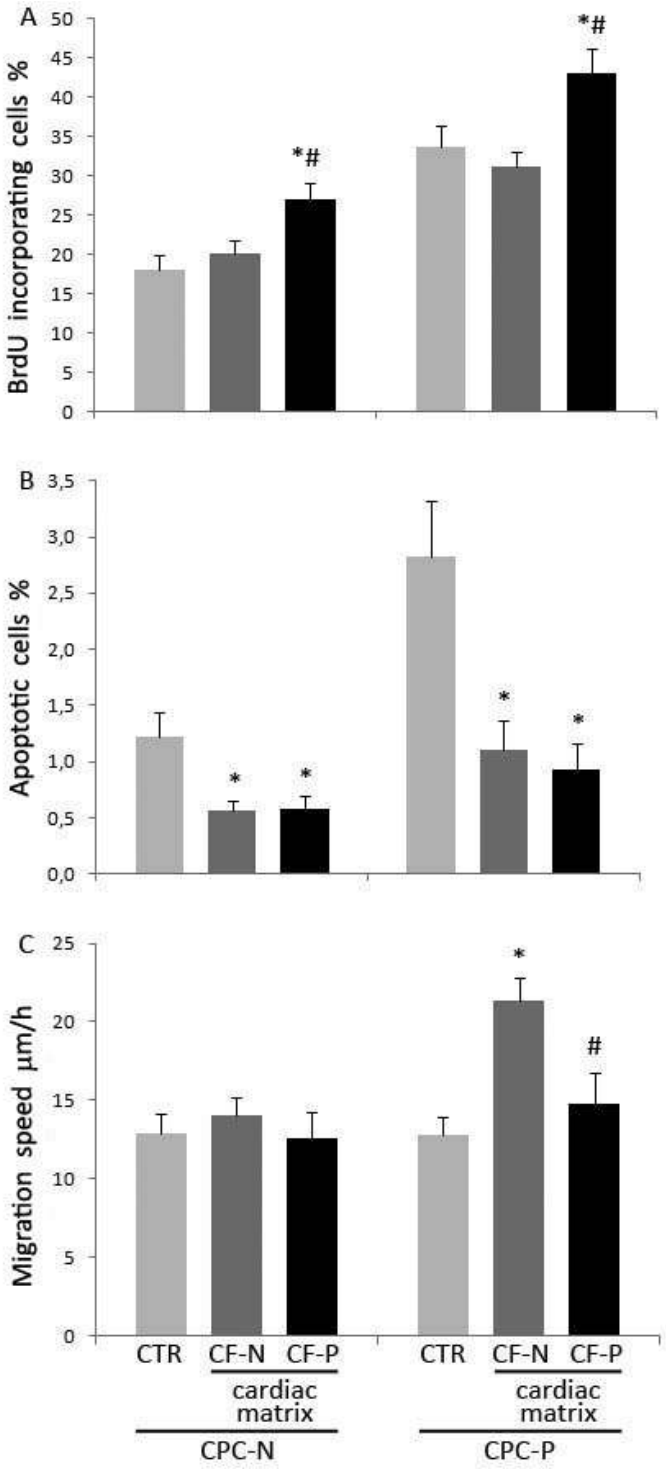

Figure 12. Proliferation (A), apoptosis (B) and migration (C) of cardiac primitive cells from normal (CPC-N) and pathological (CPC-P) hearts cultured on dishes covered with albumin (control, CTR) or cardiac matrix deposited by fibroblasts from normal (CF-N) or pathological (CF-P) heart. * $p<0,05$ vs CTR; # $p<0,05$ vs CF-N.

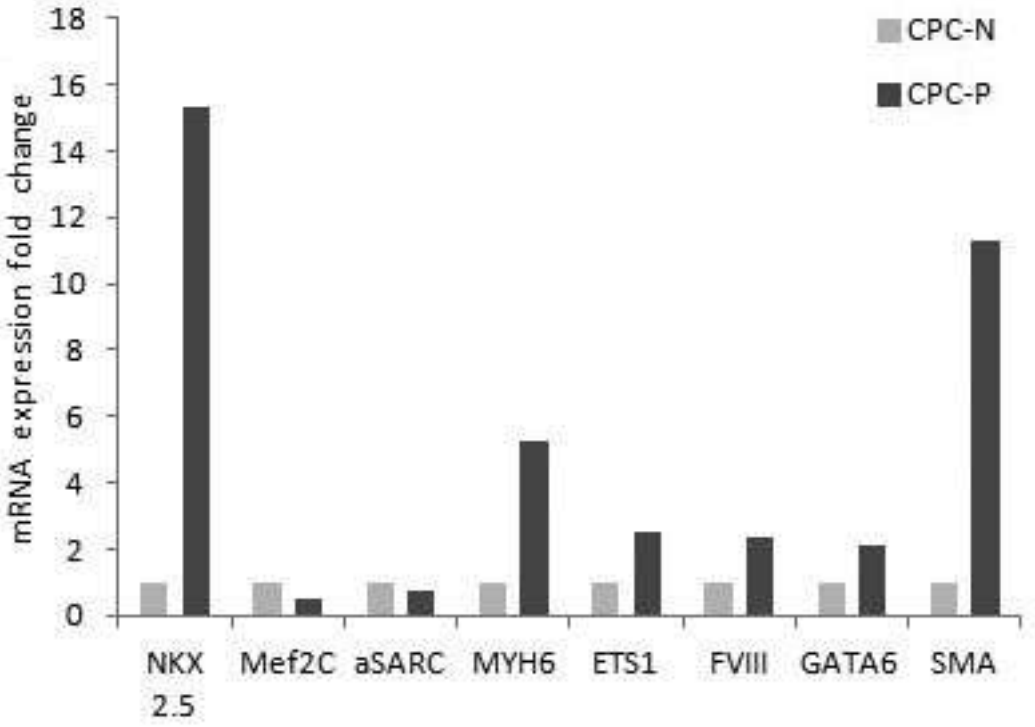

Figure 13. Differences in the expression of cardiac lineage specific genes in the population of CPC-P with respect to CPC-N cultured in standard conditions.

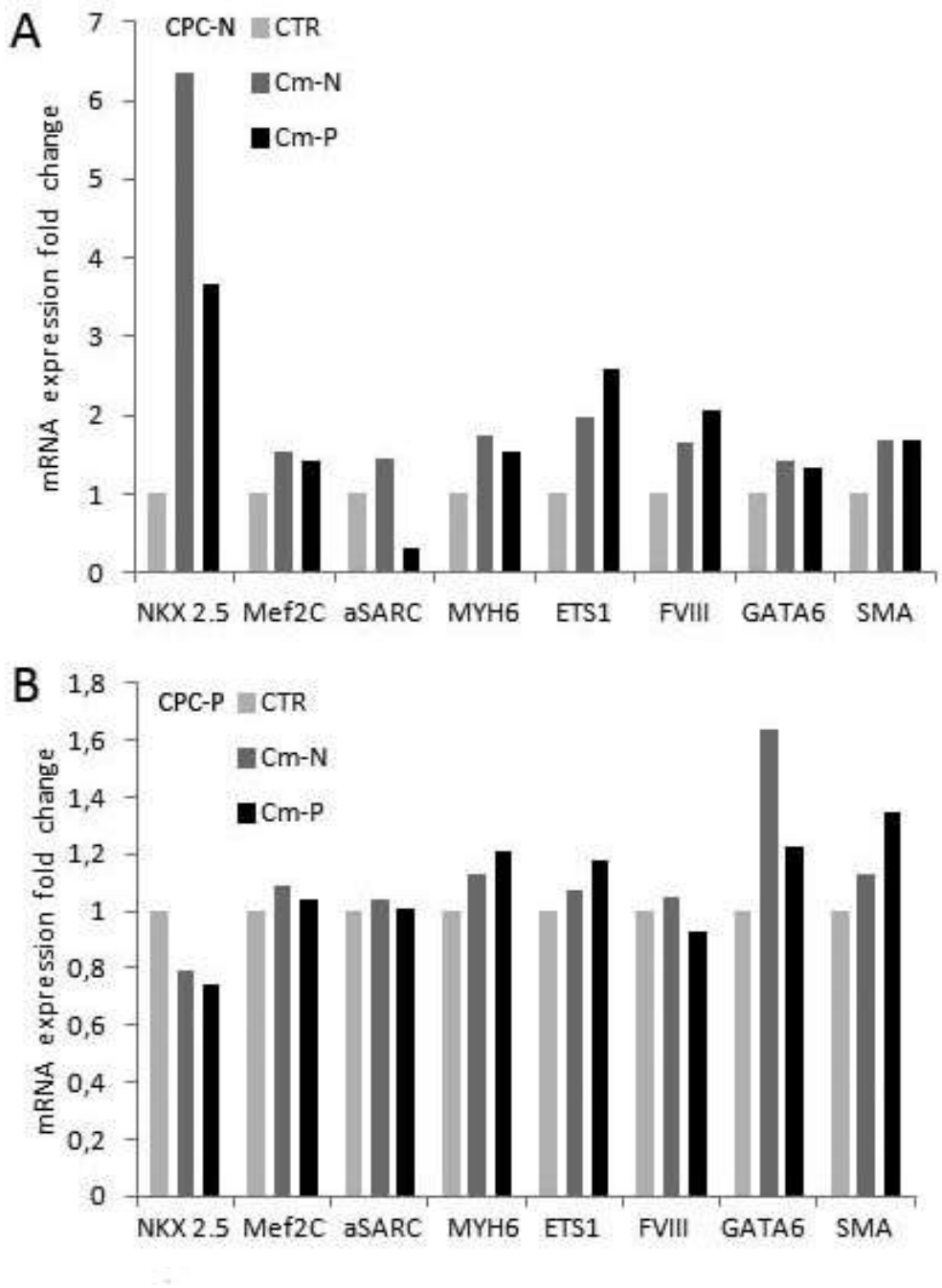

Figure 14. Differences in the expression of cardiac lineage specific genes in the population of cardiac primitive cells from (A) normal (CPC-N) or (B) pathological (CPC-P) heart cultured in the presence of cardiac matrix derived from fibroblasts from adult human normal (Cm-N) or pathological (Cm-P) heart or with respect to standard (CTR) conditions, as evaluated by RT-PCR.

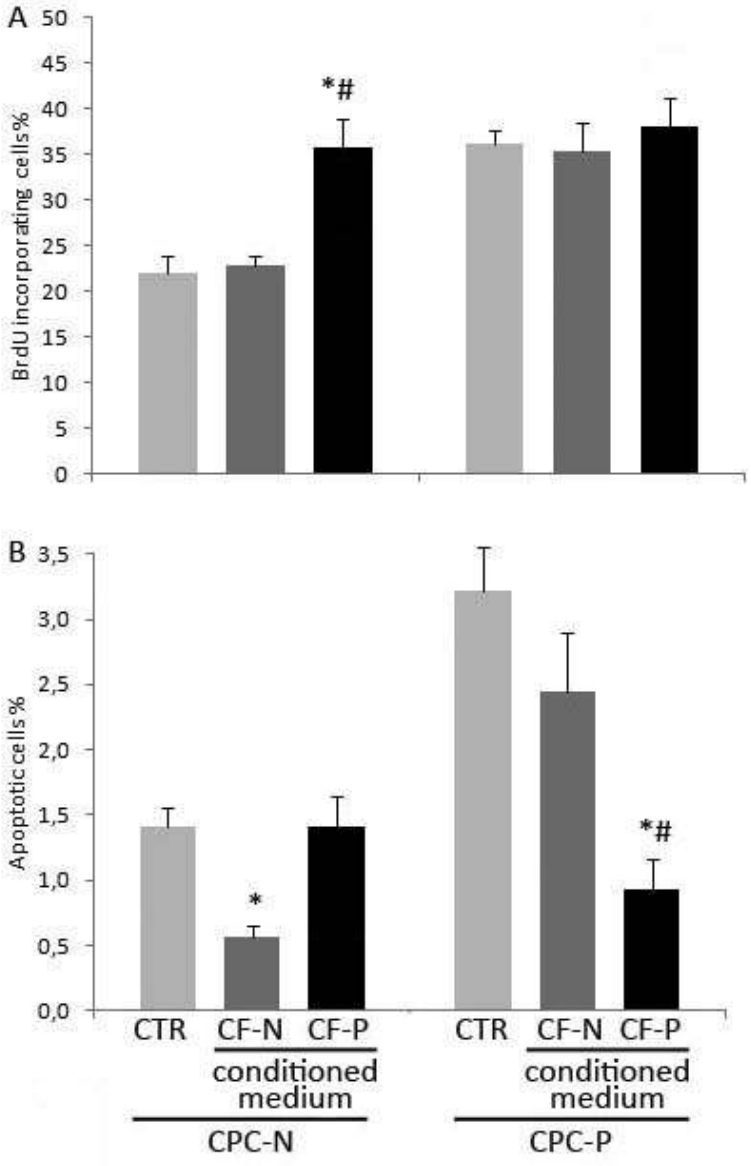

Figure 15. Proliferation (A) and apoptosis (B) of CPC-N and CPC-P cultured in the presence of medium conditioned by CF-N- and CF-P. * $p<0,05$ vs CTR; # $p<0,05$ vs CF-N.

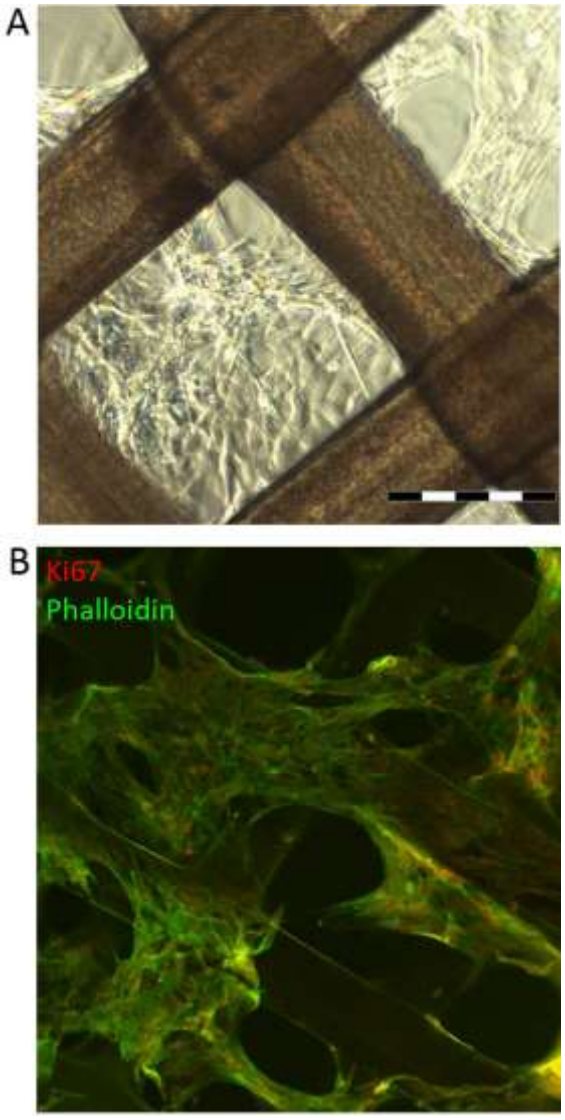

Figure 16. Representative images of polyurethane bi-layered scaffold seeded with cardiac primitive cells observed at phase contrast microscope (A) and after immunofluoresent staining of actin filaments and Ki67 (B).

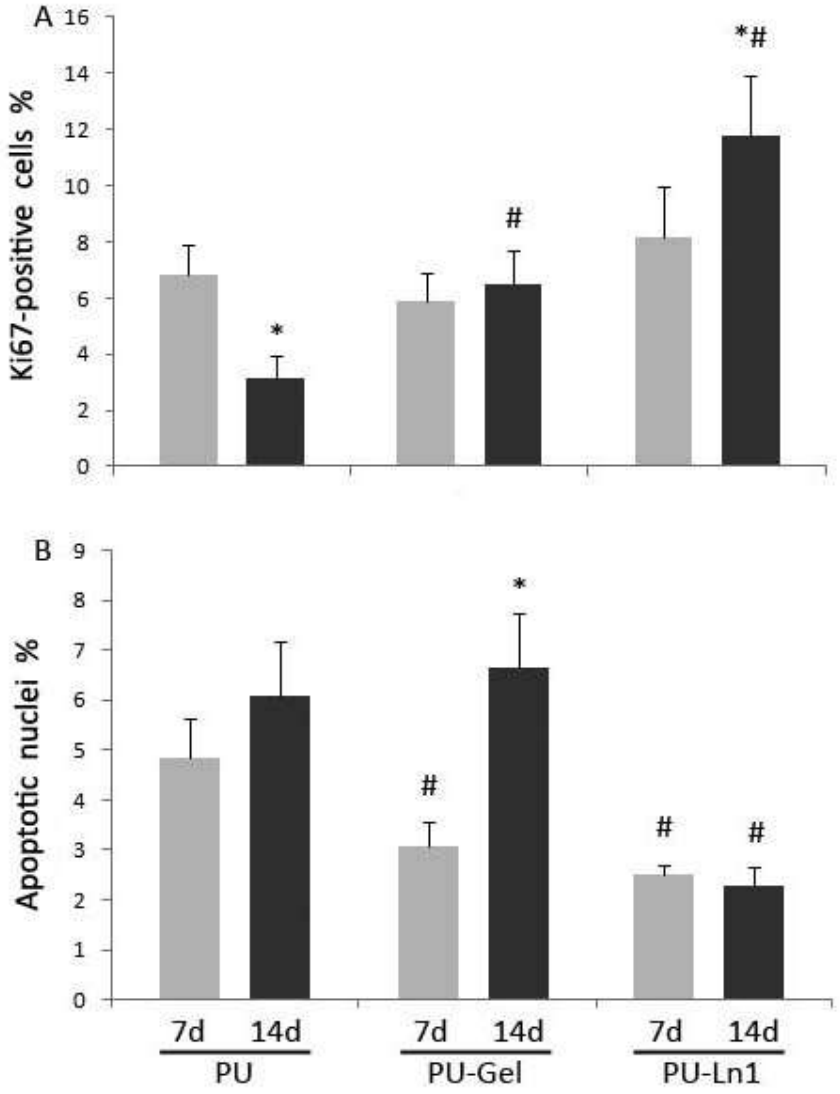

Figure 17. Proliferation (A) and apoptosis (B) of CPC-P cultured on polyurethane control scaffold (PU), polyurethane scaffold functionalized with gelatin (PU-Gel), or polyurethane scaffold functionalized with laminin-1 (PU-Ln1) for 7 or 14 days. * $p<0,05$ vs 7d; # $p<0,05$ vs PU.

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Acknowledgements

Thanks to all those people who helped me to be what I am today.

I would like to express my sincere gratitude to Prof. Stefania Montagnani, who gave me the opportunity to learn this practical knowledge, being for me a pillar more than an ordinary mentor.

My special and sincere thanks to Dr Daria Anna Nurzynska, who is much more to me than just a tutor. I hope I will prove worthy of all the trust she put in me.
I am deeply grateful to Dr Franca Di Meglio and Dr Clotilde Castaldo, who supported me in my PhD studies, allowing me to learn and teaching me how to work.
I thank them for their constant willingness and, over all, for their great tollerance. I will always be grateful for their friendship proved day by day. I'm glad to affirm that their knowledge has been precious for my professional and personal growth.

I am even more grateful to my parents, my sister and my brother, supporting me and believing in my capabilities, they gave me the chance to take on this path, encouraging me in dark moments too.

Last but not least, I would like to express my thanks to those people who worked hard to achieve this scientific goal.